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<b>(54) Title:</b> VARIABLE FRAGMENTS OF IMMUNOGLOBULINS - USE FOR THERAPEUTIC OR VETERINARY PURPOSES			
<b>(57) Abstract</b> <p>The present invention relates to fragments, especially variable fragments of immunoglobulins which are by nature devoid of light chains, these fragments being nevertheless capable of exhibiting a recognition and binding activity toward specific antigens. The present invention further relates to the use of such immunoglobulin fragments formed of at least one heavy chain variable fragment or derived therefrom, for therapeutic or veterinary purposes and especially for passive immunotherapy or serotherapy.</p>			

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Variable fragments of immunoglobulins -  
Use for therapeutic or veterinary purposes

10       The present invention relates to fragments, especially variable  
fragments of immunoglobulins which are by nature devoid of light chains  
these fragments being nevertheless capable of exhibiting a recognition and  
binding activity toward specific antigens. These fragments of  
immunoglobulins can for example be obtained by the expression in host  
15       cells for example in prokaryotic cells or eukaryotic cells of nucleotide  
sequences obtained from animals naturally expressing so-called "two-  
chain immunoglobulins", for instance from animals of the camelid family.

          The present invention further relates to the use of such  
immunoglobulin fragments formed of at least one heavy chain variable  
20       fragment or derived therefrom, for therapeutic or veterinary purposes and  
especially for passive immunotherapy or serotherapy.

          Functional immunoglobulins devoid of light polypeptide chains  
termed «two-chain immunoglobulin » or « heavy-chain immunoglobulin »  
have been obtained from animals of the family of camelids and have been  
25       described in an international patent application published under number  
WO 94/04678, together with two publications, especially Hamers-  
Casterman et al, 1993 and Muyldermans et al, 1994).

          The isolation and characterization of these immunoglobulins,  
together with their cloning and sequencing have been described in the  
30       above referenced documents which are incorporated by reference in the  
present application.

          According to WO 94/04678 it has been established that different  
molecules can be isolated from animals which naturally produce them,  
which molecules have functional properties of the well known  
35       four-chain immunoglobulins these functions being in some cases related to

5 structural elements which are distinct from those involved in the function of four-chain immunoglobulins due for instance to the absence of light chains.

These immunoglobulins having only two chains, neither correspond to fragments obtained for instance by the degradation in particular the enzymatic degradation of a natural four-chain model immunoglobulin, nor  
10 correspond to the expression in host cells, of DNA coding for the constant or the variable regions of a natural four-chain model immunoglobulin or a part of these regions, nor correspond to antibodies produced in lymphopaties for example in mice, rats or human.

The immunoglobulins devoid of light chains are such that the  
15 variable domains of their heavy chains have properties differing from those of the four-chain immunoglobulin variable heavy chain ( $V_H$ ). For clarity reasons, this variable domain according to the invention will be called  $V_{HH}$  in this text to distinguish it from the classical  $V_H$  of four-chain immunoglobulins. The variable domain of a heavy-chain immunoglobulin  
20 according to the invention has no normal interaction sites with the  $V_L$  or with the  $C_H1$  domain which do not exist in the heavy-chain immunoglobulins. It is hence a novel fragment in many of its properties such as solubility and conformation of main chains. Indeed the  $V_{HH}$  of the invention can adopt a three-dimensional organization which distinguishes  
25 from the three-dimensional organization of known four-chain immunoglobulins according to the description which is given by Chothier C. and Lesk A.M, (1987- J.Mol. Biol. 197, 901-917).

According to the results presented in patent application WO 94/04678, the antigen binding sites of the isolated immunoglobulins,  
30 naturally devoid of light chains are located on the variable region of their heavy chains. In most cases, each heavy chain variable region of these two-chain immunoglobulins can comprise an antigen binding site.

A further characteristic of these two-chain immunoglobulins is that their heavy polypeptide chains contain a variable region ( $V_{HH}$ ) and a

5 constant region ( $C_H$ ) according to the definition of Roitt et al but are devoid of the first domain of the constant region is called  $C_{H1}$ .

These immunoglobulins of the type described hereabove can comprise type G immunoglobulins and especially immunoglobulins which are termed immunoglobulins of class 2 (IgG2) or immunoglobulins of class  
10 3 (IgG3), according to the classification established in patent application WO 94/04678 or in the publication of Muyldermans et al (Protein Engineering Vol.7, N°9, pp 1129-1135-1994).

The absence of the light chain and of the first constant domain lead to a modification of the nomenclature of the immunoglobulin fragments  
15 obtained by enzymatic digestion, according to Roitt et al.

The terms Fc and pFc on the one hand, Fc' and pFc' on the other hand corresponding respectively to the papain and pepsin digestion fragments are maintained.

The terms Fab, F(ab)<sub>2</sub>, F(ab')<sub>2</sub>, Fabc, Fd and fv are no longer  
20 applicable in their original sense as these fragments have either a light chain, the variable part of the light chain or the CH<sub>1</sub> domain.

The fragments obtained by papain digestion or by V8 digestion, composed of the V<sub>HH</sub> domain of the hinge region will be called FV<sub>HHh</sub> or F(V<sub>HHh</sub>)<sub>2</sub> depending upon whether or not they remain linked by the  
25 disulphide bonds.

The immunoglobulins referring to the hereabove given definitions can be originating from animals especially from animals of the camelid family. These heavy-chain immunoglobulins which are present in camelids are not associated with a pathological situation which would induce the  
30 production of abnormal antibodies with respect to the four-chain immunoglobulins. On the basis of a comparative study of old world camelids (Camelus bactrianus and Camelus dromaderius) and new world camelids (for example Lama Paccos, Lama Glama, and Lama Vicugna) the inventors have shown that the immunoglobulins devoid of light polypeptide  
35 chains are found in all species. Nevertheless differences may be apparent

5 in molecular weight of these immunoglobulins depending on the animals. Especially the molecular weight of a heavy chain contained in these immunoglobulins can be from approximately 43 kd to approximately 47 kd, in particular 45 kd.

Advantageously the heavy-chain immunoglobulins of the invention  
10 are secreted in blood of camelids.

The variable fragments of heavy chains of Immunoglobulins devoid of light chains can be prepared starting from immunoglobulins obtainable by purification from serum of camelids according to the process for the purification as described in detail in the examples of WO 94/04678. The  
15 variable fragments can also be obtained from heavy-chain immunoglobulins by digestion with papain or V8 enzymes.

These fragments can also be generated in host cells by genetic engineering or by chemical synthesis. Appropriate host cells are for instance bacteria (e.g. E. coli) eucaryotic cells including yeasts or animal  
20 cells including mammalian cells, or plant cells.

The observation by the inventors that Camelidae produce a substantial proportion of their functional immunoglobulins as a homodimer of heavy chains lacking the C<sub>H</sub>1 domain and devoid of light chains (Hamers-Casterman et al, 1993), led to the proposal of having recourse to an  
25 immunized camel to generate and select single variable antibody fragments (V<sub>HH</sub>) and furthermore give access to the corresponding nucleotide sequences.

Cloned camel single V<sub>HH</sub> fragments were displayed on bacteriophages for selection and in bacteria for the large scale production  
30 of the soluble proteins, and were shown to possess a superior solubility behaviour and affinity properties compared to the mouse or human V<sub>H</sub> equivalents (Muyldermans et al, 1994). Following this strategy, one would obtain small ligand binding molecules (MW around 16,000 D) which are not hindered by the presence of an oligopeptide linker (Borrebaeck *et al.*,  
35 1992) or not inactivated by the disassembly of the VH-VL complex

- 5 (Glockshuber *et al.*, 1990). The camel  $V_{HH}$  fragments have the additional advantage that they are characteristic of the heavy chain antibodies which are matured in vivo in the absence of light chains.

The inventors have obtained evidence that variable fragments of high chains of immunoglobulins devoid of light chains can display an effective therapeutic activity when they are generated against a determined antigen.

To develop this technology of preparing and identifying usefull camel  $V_{HH}$  fragments, it is critical (i) that camels can be immunized with a variety of antigens, (ii) that the camel  $V_{HH}$  genes can be cloned and expressed on filamenteous phages and in E.coli for easy selection with the immobilized antigen by panning, (iii) that the expressed camel  $V_{HH}$ 's are properly folded, and (iv) that they have good solubility properties and possess high affinities and specificities towards their antigen.

Camel  $V_{HH}$  genes derived from the heavy chain immunoglobins lacking the light chains were previously cloned and analysed (Muyldermans *et al.*, 1994). A comparison of the amino acid sequences of these camel  $V_{HH}$  clones clearly showed that the key features for preserving the characteristic immunoglobulin fold are all present. The specific amino acid replacements observed in the camel  $V_{HH}$  clones could correlate with the absence of the VL (variable light chains) and the functionality of the camel single  $V_{HH}$  domain (Muyldermans *et al.*, 1994).

The invention thus relates to a variable fragment ( $V_{HH}$ ) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:

- 30 - treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,

- 5 - contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a
- 10 FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3 ' or 5'-CGCCATCAAGGTACCAGTTGA-3 '
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- 15 - recovering amplified DNA corresponding to bands of different size orders including:
- . a band of around 750 basepairs which is the amplified product of the variable heavy chain ( $V_H$ ), CH1, hinge and part of CH2 region of a four-chain immunoglobulin,
  - 20 . a band of around 620 basepairs which is the amplified product of the variable heavy-chain ( $V_{HH}$ ), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
  - . a band of around 550 basepairs which is the amplified product of the variable heavy-chain ( $V_{HH}$ ), short hinge, and part of the CH2 of the camel
- 25 two-chain immunoglobulin IgG3,
- purifying the two shortest bands of around 620 and 550 basepairs from agarose gel, for example by Gene Clean,
  - recovering the amplified DNA fragments containing nucleotide sequences encoding the  $V_{HH}$  fragments,
  - 30 - digesting the amplified products with restriction enzymes having target sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
  - recovering the digested amplified DNA fragments,
  - ligating the amplified DNA fragments to a phasmid vector, for example in
- 35 a pHEN4 vector, in conditions allowing the expression of the amplified



- 5 fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- 10 - infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant
- 15 phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
  - submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- 20 - eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
  - recovering the virions and testing them for their binding activity against
- 25 the antigen of interest, for example by ELISA,
- recovering the phagemid virions having the appropriate binding activity,
  - isolating the nucleotide sequence contained in the phasmid vector and capable of being expressed on the phagemid virions as a  $V_{HH}$  aminoacid sequence having the appropriate binding activity.
- 30 In a preferred embodiment of the invention, the variable  $V_{HH}$  fragments are obtainable by adding to the hereabove described amplification step of the cDNA with BACK and FOR primers (p1), a further amplification step with a BACK primer corresponding to the oligonucleotide sequence which has been described hereabove (back p1) and the FOR
- 35 primer (for p2) having the following nucleotide sequence: 5'- CG ACT AGT

5     GCG GCC GCG TGA GGA GAC GGT GAC CTG-3'. Not and BstEII sites which can be used for cloning in the pHEN4 vector have been underlined. This FOR primer allows hybridization to the codon position of framework 4 (FR4) region of the V<sub>HH</sub> nucleotide sequences (amino acid position 113-103).

10         According to another variant of the process described, this additional amplification step can replace the amplification step which has been described with BACK primer and a FOR primer having respectively the following nucleotide sequences:

5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

15     5'-CGCCATCAAGGTACCGTTGA-3'                     or

5'-CGCCATCAAGGTACCAGTTGA-3'

The restriction sites have been underlined.

           In another embodiment of the invention the amplification step of the synthesized cDNA is performed with oligonucleotide primers including  
20     hereabove described BACK primer and FOR primer having the following sequences:

FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'

FOR primer 4: 5'- TTC ATT CGT TCC TGA GGA GAC GGT -3'

25

           According to this latter embodiment, the V<sub>HH</sub> fragments of the invention are immediately and specifically amplified by a single amplification (for instance PCR reaction) step when the mixture of FOR primers is used.

30         These latter primers hybridize with the hinge/framework 4 and short hinge/framework 4 respectively. Each of these FOR primers allows the amplification of one IgG class according to the classification given in patent application WO 94/04678.

           The variable V<sub>HH</sub> fragments corresponding to this definition can also  
35     be obtained from other sources of animal cells, providing that these

5 animals are capable of naturally producing immunoglobulins devoid of light chains according to those described in the previous patent application WO 94/04678.

These variable fragments ( $V_{HH}$ ) can also be obtained by chemical synthesis or by genetic engineering starting from DNA sequences which  
10 can be obtained by the above described process.

The variable fragment of a heavy chain of an immunoglobulin devoid of light chains according to the preceding definitions is specifically directed against an antigen against which the animal has been previously immunized, either by natural contact with this antigen or by administration  
15 of this antigen in order to generate an immune response directed against it.

The process which is proposed hereabove to prepare a nucleotide sequence coding for the variable fragments of the invention contains steps of phage display library construction which allow the selection of nucleotide sequences coding for variable fragments of heavy chains having the  
20 desired specificity.

According to one preferred embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin is obtainable from an animal having been previously immunized with a toxin, especially a toxin of a bacteria or a part of this toxin sufficient to enable the production  
25 of immunoglobulins directed against this toxin and especially immunoglobulins devoid of light chains.

According to another embodiment of the invention, the variable fragments of a heavy chain of a immunoglobulin is obtainable from an animal having been previously immunized with substances contained in  
30 venom of animals.

The antigen used for immunization of the animals is usually under a non toxic form.

The variable fragments according to the invention can be derived from immunoglobulins belonging to different classes especially belonging

- 5 to IGg2 or IGg3 immunoglobulin classes, according to the classification given in patent application WO/04678.

In a preferred embodiment of the invention, the variable fragment of a heavy-chain of an immunoglobulin devoid of light chains is directed against the tetanus toxin of Clostridium tetani or against a fragment thereof.

The variable fragments of heavy chains of immunoglobulins devoid of light chains can be also generated against toxins or part thereof from pathogenic organisms such as bacteria and especially can be chosen among the toxins or toxoids of the following bacteria: Clostridium,  
 15 especially Clostridium Botulinum or Clostridium Perfringens,  
Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis,  
Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella,  
Shigella, Listeria.

Other antigens appropriate for the preparation of the V<sub>HH</sub> fragments of the invention can be obtained from the following organism: anemonies,  
 20 coral, jellyfish, spiders, bees, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

According to another embodiment of the invention, the variable fragment V<sub>HH</sub> of a heavy chain of an immunoglobulin devoid of light chains  
 25 is characterized in that it comprises the following aminoacid sequences:

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu

ArgLeuSerCysAla(Ala/Thr)SerGly(CDR1)Trp(Phe/Tyr)ArgGlnAlaProGlyLys

30

Glu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)(CDR2)Arg(Phe/Leu)ThrIleSer(Arg/

Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)TyrLeu(Gln/Leu)MetAsnSerLeu

35 (Lys/Glu)ProGluAspThrAla(Val/Met/Ile)TyrTyrCysAlaAla(CDR3)TrpGlyGln

5

GlyThrGlnValThrValSerSer or

(Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu

10 ArgLeuSerCysAla(Ala/Tlu)SerGly(Ala,Thr,Ser,Ser/Tyr,Thr,Ile,Gly)(**CDR1**)

Trp(Phe/Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)

(**CDR2**)Arg(Phe/Leu)ThrIleSer(Arg/Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)

15

TyrLeu(Gln/Leu)MetAsnSerLeu(Lys/Glu)ProGluAspThrAla(Val/Met/Ile)Tyr

TyrCysAlaAla(**CDR3**)TrpGlyGlnGlyThrGlnValThrValSerSer,

20 wherein CDR1, CDR2 and CDR3 represent variable amino acid sequences providing for the recognition of a determined epitope of the antigen used for the immunization of Camelids, CDR1, CDR2 and CDR3 sequences comprising from 5 to 25 amino acid residues preferably CDR1 contains from 7 to 12 amino acid residues, CDR2  
25 contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

The camel V<sub>HH</sub> specific amino acid residues Ser 11, Phe 37, Glu 44, Arg 45, Glu 46, Gly 47 are underlined.

30 One preferred variable fragment according to the invention is encoded by a nucleotide sequence present in recombinant phasmid pHEN4- $\alpha$ TT2(WK6) deposited at the BCCM/LMBP (Belgium) under accession number LMBP3247.

The pHEN4 $\alpha$ TT2 (described on Figure 2) is a phasmid carrying a PelB leader signal, a camel V<sub>HH</sub> gene of which the protein binds tetanus

5 toxoid, a decapeptide tag (from ImmunoZAP H of Stratacyte) and gene IIIp of M13 in the pUC 119 polylinker between the HindIII and EcoRI sites. This phasmid was transformed in E. coli WK6 cells.

A specific variable fragment according to the invention is for instance characterized in that it comprises the following  $\alpha$ TT1 aminoacid  
10 sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly**Ser**ValGlnAlaGlyGlySerLeuArgLeu

SerCysAlaAlaSerGlyGlyGlnThrPhe**AspSerTyrAlaMetAla**TrpPheArgGln  
15

AlaProGlyLysGluCysGluLeuValSer**SerIle**lleGly**AspAspAsnArgAsnTyr**

**AlaAspSerValLysGly**ArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

20 LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln**LeuGly**

**SerAlaArgSerAlaMetTyrCys**AlaGlyGlnGlyThrGlnValThrValSerSer

25 According to another preferred embodiment of the present invention, the variable fragment comprises the following  $\alpha$ TT2 aminacid sequence :

GluValGlnLeuGlnAlaSerGlyGlyGly**Ser**ValGlnAlaGlyGlySerLeuArgLeu

30 SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu**Arg**GluGlyValAla**GlyIle**SerSer**GlyGlySerThrThrAlaTyr**

**SerAspSerValLysGly**ArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr  
35

5 LeuLeulleAspAsnLeuGlnProGluAspThrAlalleTyrTyrCysAlaGly**ValSer**

**GlyTrpArgGlyArgGlnTrpLeuLeuLeuAla**GluThrTyrArgPheTrpGlyGlnGly

**ThrGlnValThrValSerSer**

10

In a preferred embodiment of the invention, the variable V<sub>HH</sub> fragment of the invention is altered in order to diminish its immunogenic properties. Such a modification can lead to an alternated immunological reaction against the V<sub>HH</sub> fragments of the invention when they are  
15 administered to a host either human or animal, for passive immunoprotection for example.

The invention further relates to a pharmaceutical composition comprising an immunoglobulin heavy chain variable fragment according to those which have been defined hereabove, in admixture with a  
20 physiologically acceptable vehicle.

Such pharmaceutical composition can be used for the treatment by passive immunisation, of infections or acute intoxications by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia,  
25 Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, bees, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

The present invention further relates to nucleotide sequences coding for a variable fragment (V<sub>HH</sub>) of a heavy chain of an immunoglobulin devoid of light chains, obtainable by the process which has been described  
30 hereabove.

Specific nucleotide sequences are those corresponding to  $\alpha$ TT1 and  $\alpha$ TT2 as described on figures 4A and 4B.

5           According to an embodiment of the invention, a preferred nucleotide sequence is the sequence contained on phasmid pHEN4- $\alpha$ TT2 deposited at the BCCM/LMBP collection in Belgium on January 31, 1995 under no. LMBP3247.

10           The invention further provides means for the preparation of bivalent or even multivalent monospecific DNA constructs of variable fragments of an immunoglobulin devoid of light chains and their expression products. It thus gives access to the preparation of monovalent bispecific or multispecific variable constructs obtained from sequences encoding  $V_{HH}$  fragments combined with a linker sequence. Bivalent monospecific  
15           constructs contain 2 nucleotide sequences coding for  $V_{HH}$  fragments directed against the same antigen or epitope. Monovalent bispecific constructs contain on one molecule one nucleotide sequence coding for a  $V_{HH}$  fragment directed against one antigen or epitope and another nucleotide sequence coding for a fragment directed against another  
20           antigen or epitope.

          The corresponding expression products (protein constructs) can be obtained by genetic engineering especially by expression in host cells, like bacteria (e.g. *E. coli*) or eukaryotic cells, of the above DNA constructs.

25           Accordingly a variable fragment of the  $V_{HH}$  type having a determined antigen specificity, can be linked to at least one further variable fragment  $V_{HH}$  having a determined similar or different specificity in terms of antigen- and/or epitope specificity.

          The obtained constructs (in terms of expression products) and especially the bivalent monospecific constructs advantageously offer  
30           means to improve the affinity for the antigen(s) against which they are obtained.

          The linker sequence between the  $V_{HH}$  fragments can be for example a sequence corresponding to the coding sequence of the hinge domain of immunoglobulin devoid of light chains (e.g. the long hinge domain) as



5 described by (Hamers-Casterman C. et al, 1993) or a sequence derived therefrom.

As an example, in order to ligate these two variable coding sequences of  $V_{HH}$  fragments to obtain monovalent bispecific construct, the sequence coding for the hinge and  $CH_2$  domains, especially coding for the  
10 long hinge and  $CH_2$  domains of an Immunoglobulin devoid of light chains can be used. These domains have been described in WO 94/04678.

As another example, for instance for the preparation of bispecific or multispecific DNA constructs, the sequence used as linker between the  $V_{HH}$  fragments is derived from the coding sequence of the hinge and is devoid  
15 of the terminal part containing nucleotides coding for the cysteine residue, or more generally devoid of the codons enabling dimerisation of the  $V_{HH}$  fragment.

Preferred linkers include: the sequence starting at nucleotide 400 and ending at nucleotide 479 or between nucleotides 479 and 486 of the  
20 nucleotide sequence disclosed on figure 15 or the sequence starting at nucleotide 400 and ending at nucleotide 495 or between nucleotides 487 and 495 of the nucleotide sequence of figure 15.

The linkers can be for instance obtained by digestion of a plasmid containing the coding sequence for the  $V_{HH}$ , hinge and  $CH_2$  domains of an  
25 immunoglobulin devoid of light chains, with Bst EII and XmnI (or KpnI) endonucleases and further amplification of the sequence with primers annealing to each end of the hinge coding sequence as described above and illustrated in the examples.

As an example, constructs (monovalent or multivalent, monospecific  
30 or multispecific) can be obtained having a specificity with respect to two or more different toxins or generally antigens of different pathogen organisms including bacteria, viruses...

The invention also relates to a process for the preparation of monovalent bispecific constructs of variable fragments of a heavy chain of  
35 an immunoglobulins which comprises the following steps:

5           a)     ligating a nucleotide sequence coding for a variable  $V_{HH}$  fragment having a determined antigen- or epitope- specificity to a linker nucleotide sequence to form a  $V_{HH}$ -linker fragment;

          b)     ligating the formed nucleotide sequence coding for the  $V_{HH}$ -  
10 linker fragment to a nucleotide sequence coding for another  $V_{HH}$  fragment having a different antigen- and/or epitope-specificity,

          wherein the linker sequence contains the nucleotide sequence coding for part of a hinge domain wherein the codons responsible for the dimerisation of the  $V_{HH}$  fragments especially by formation of a disulfide bridge between the last cysteine residues within the hinge domain are  
15 deleted.

          According to a preferred embodiment, additional steps of ligation are performed with sequences coding for variable fragments ( $V_{HH}$  fragments) having the same specificity or a different specificity with respect to the above fragments.

20           In such a case the  $V_{HH}$ -hinge linker -  $V_{HH}$  fragment coding sequences recovered from step b) must be digested so as to produce a nucleotide sequence having the following structure hinge linker -  $V_{HH}$ . In accordance  $(V_{HH} - \text{hinge linker})_n$  coding sequences are obtained wherein n is a number higher than 2.

25           Preferably, the sequence encoding the hinge domain preferably the long hinge domain of the immunoglobulins devoid of light chains is the nucleotide sequence comprising or corresponding to nucleotides 400 to 479 or up to nucleotides 486 of the sequence of figure 15.

          In a particular embodiment of the process for the preparation of  
30 bivalent or multivalent monospecific or multispecific constructs, the  $V_{HH}$  fragment coding sequence linked to a nucleotide sequence encoding the hinge domain has to be amplified. Oligonucleotide primers have been defined which permit the amplification of the sequence of interest. These oligonucleotides anneal respectively with their 3' end to the beginning of

5 the V<sub>HH</sub> gene or coding sequence and to the terminal part of the hinge coding sequence. Appropriate primers are for instance:

**A4** (Sf I site underlined):

5'CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGA(G,T)GT(G,C)C  
AGCT-3'

10

**AM007:**

5'GGCCATTTGCGGCCGCATTCCATGGGTTCAGGTTTTGG-3'

15 These chosen primers contain target sequences for specific endonucleases, thus allowing the cloning of the digestion products of the amplified fragments in a suitable vector.

The obtained DNA constructs are then used to transform host cells, for instance E. coli and the expressed proteins are then isolated and  
20 purified. The expression products of these DNA constructs are within the scope of the invention.

The heavy-chain antibodies, such as those derived from camel, and their fragments present clear advantages over other antibodies or fragments thereof derived from other animals. These are linked to the  
25 distinctive features of the heavy chain antibodies and in particular the novel fragments which can be produced by proteolytic cleavage within the hinge of these heavy-chain antibodies to generate the V<sub>HH</sub> and the (V<sub>HHh</sub>)<sub>2</sub> fragments. The V<sub>HH</sub> domain of a heavy chain has distinct genetic entities which confer properties of solubility not found in VH fragments derived from  
30 conventional antibodies. This property, in addition to its small size and to the fact that the amino acid sequence of the framework region is very homologous to that of human, ensures a minimum of immunogenicity. These properties would allow repetitive treatment with heavy chain V<sub>HH</sub> fragments for passive immunisation or antibody therapy. As mentioned  
35 above, V<sub>HH</sub> and the (V<sub>HHh</sub>)<sub>2</sub> fragments can easily be produced by

- 5    proteolytic cleavage of camel immunoglobulins or via recombinant DNA technology.

          The most important field of passive immunisation is intoxication due to bacterial toxins and in particular acute intoxication or intoxication due to drug resistant bacteria. Passive immunisation or treatment by antibodies is  
10    justified in those cases where vaccination is unpractical or its effects short-lived. They are particularly justified for acute intoxication which if left untreated would have lethal or crippling effects.

          The following list of indications is non-exhaustive:

- Tetanos due to infection by *Clostridium tetani* is an important post-trauma  
15    infection and current immunisations are not long lasting. It is also important in the veterinary field.
- Botulism due to ingestion of toxins produced by *Clostridium Botulinum* and related species.
- Gangrene due to infection by *Clostridium*.
- 20    - Necrotic Enteritis and Enterotoxemia in humans and livestock due to *Clostridium Perfringens* ingestion.
- Food poisoning due to Staphylococcal endotoxins in those cases where antibiotics are not recommended.
- *Pseudomonas* infection refractory to antibiotic treatment and in particular  
25    ocular infections where rapid intervention is warranted.
- Diphtheria toxin infection
- *Pasteurella* and *Yersinia* infection causing lethal outcomes in human and livestock.
- Anthrax toxin produced by *Bacillus Anthrax* and responsible for one of  
30    the five major livestock diseases.
- Infections due to other bacterial agents such as *Neisseria* or viral agents.

          Furthermore, the relative resistance of the  $V_{HH}$  fragment to proteolytic cleavage by digestive enzymes (e.g. pepsin, trypsin) offer the possibility of treatment against important gut pathogens, such as Vibrio

5    cholera and other vibrios, enterotoxic E.Coli, Salmonella species and Shigella or pathogens ingested with food such as Listeria.

Another major target for immunotherapy is in the treatment of intoxication due to bites or contact with toxic invertebrates and vertebrates. Among the invertebrates are sea anemonies, coral and jellyfish, spiders,  
10    bees and wasps, scorpions. In the vertebrates, the venomous snakes are of particular importance and in particular those belonging to the families of Viperidae, Crotalidae and lapidea.

Passive immunisation with partially purified immunoglobulins from immunized animals are already being used. In developing countries,  
15    antitetanos and antidiphtheria antisera are still produced on a very large scale, usually in horses. Anti-venom antibodies are produced, although on a much smaller scale, against venoms, especially snake venoms.

Another field of application is in combination with the therapeutic use of toxins in medical or surgical practice where neurotoxins such as  
20    botulinum toxin are increasingly used.

The invention also relates to the oligonucleotide primers described hereabove, either alone or in kits.

Other characteristics of the invention will appear from the figures and the examples which are described hereafter.

25    **FIGURE 1:** 1% agarose gel electrophoresis of the PstI/BstEII digested PCR amplification product of the camel V<sub>HH</sub> gene (lanes 1 and 2) next to the 123 bp ladder of BRL used as a size marker (lane 4). The PCR product comigrates with the 3<sup>rd</sup> band of the marker, 369 bp in length.

**FIGURE 2:** Map of the pHEN4 with the nucleotide sequence of the  
30    V<sub>HH</sub> cloning site shown in the lower part of the figure. The PstI and BstEII sites can be used to clone the camel V<sub>HH</sub> PCR product shown in Figure 1.

**FIGURE 3:** 100 individual clones were randomly selected from the original camel V<sub>HH</sub> library (0), or after the first (1), second (2), third (3) or fourth (4) round of panning. After M13 infection the virions were tested for

5 binding activity against immobilized tetanus toxoid. The number of positive clones are shown as a function of number of pannings.

**FIGURE 4:** Nucleotide sequence and the corresponding amino acid sequence of the two identified camel  $V_{HH}$  anti tetanus toxoid clones pHEN4- $\alpha$ TT1 and pHEN4- $\alpha$ TT2. The framework Ser11, Phe37 and Arg or Cys 45 characteristic for the camel  $V_{HH}$  heavy chain antibodies (Muyldermans et al, 1994) are double underlined. The three hypervariable or CDR's according to Kabat et al, (1991) are underlined.

**FIGURE 5:** SDS-polyacrylamide gel electrophoresis of the proteins extracted from the periplasm of WK6 cultures induced with IPTG. Lane 1 & 8, protein size marker (Pharmacia) MW are (from top of to bottom) 94,000; 67,000; 43,000; 30,000; 20,100 and 14,400 D. Lanes 2 and 7 Expressed periplasmic proteins extracted from WK6 cells containing pHEN4- $\alpha$ TT2' and pHENA- $\alpha$ TT1' cloning vector. Lane 3 & 4, Purified  $V_{HH}$  domain of pHEN4- $\alpha$ TT2 at 10 and 1 microgram. Lanes 5 & 6, Purified  $V_{HH}$  domain of pHEN4- $\alpha$ TT1 at 10 and 1 microgram. The position of the expressed soluble camel VH protein is indicated with an arrow. It is clearly absent in the second lane.

**FIGURE 6:** The total periplasmic extract of 1 liter of culture of WK6 cells carrying the pHEN4- $\alpha$ TT2 was concentrated to 5 ml and fractionated by gel filtration on Superdex 75 (Pharmacia) using 150mM NaCL, 10 mM sodiumphosphate pH7.2 as eluent. The pure  $V_{HH}$  is eluted at the fractions between the arrows.

**FIGURE 7:** CD (Circular dichroism) spectrum (Absorbance versus wavelength in nm) of the purified  $V_{HH}$  domain  $\alpha$ TT2 at  $3.9 \times 10^{-6}$  M in water measured in a cuvette with a pathlength of 0.2 cm. The negative band near 217 and 180 nm and the positive band around 195 nm are characteristic for  $\beta$  structures (Johnson, 1990).

5        **FIGURE 8:** Specificity of antigen binding shown by competitive ELISA. The experiments were carried out in triplicate with the bacterial periplasmic extracts of pHEN4- $\alpha$ TT1 and pHEN4- $\alpha$ TT2.

**FIGURE 9:** Number of mice surviving after I.P injection of 100 ngr tetanus toxin (10 x LD50) or co-injection of tetanus toxin with the purified  
10     $V_{HH}$   $\alpha$ TT1,  $\alpha$ TT2 or the non-specific cVH21 (Muyldermans et al., 1994) at 4 or 40 microgram.

**FIGURE 10:** Variability plot of the camelid  $V_{HH}$  sequence (CDR3 and framework 4 regions are not included).

      The alignment of the  $V_{HH}$  amino acid sequences of camel and lama  
15    (a total of 45 sequences) was performed according to Kabat et al. The variability at each position was calculated as the number of different amino acids occurring at a given position, divided by the frequency of the most common amino acid at that position. Positions are numbered according to Kabat et al. The positions above the horizontal bar indicate the amino  
20    acids which are referred to as (CDR1) and (CDR2) in the consensus sequence.

      A variability number equal to 1 indicates a perfectly conserved amino acid at that position. The higher the variability number the more likely it will be that the amino acid at this position will deviate from the  
25    consensus sequence.

**FIGURE 11:** Nucleic acid sequence of LYS2  $V_{HH}$  and translation product thereof.

**FIGURE 12:** Nucleic acid sequence of LYS3  $V_{HH}$  and translation product thereof.

30        **FIGURE 13:** Scheme to construct the bivalent monospecific anti-LYS3 camel  $V_{HH}$ .

**FIGURE 14:** Scheme to construct the monovalent bispecific anti-LYS3-long hinge linker-anti-LYS2-Tag.

5            FIGURE 15: Nucleotide and amino acid sequence of the anti-LYS3-  
long hinge/Cys-Tag. This protein will spontaneously dimerise.  
double underlined: amino acids specific for camellid  $V_{HH}$   
Boxed: CDR's  
underlined with dashes: long hinge linker  
10 underlined: Tag  
Boxed S: Cysteine which is involved in the interdomain disulfide bond.

FIGURE 16: Nucleotide and amino acid sequence of the anti-LYS3-  
long hinge linker-anti-LYS2-Tag polypeptide.  
For underlining and boxes see legend figure 15.

15

**EXAMPLE I: GENERATION OF SPECIFIC CAMEL  $V_{HH}$  FRAGMENTS  
AGAINST TETANUS TOXOID**

          In this application, results are presented, which prove the feasibility  
of generating specific camel  $V_{HH}$  fragments with demonstrated folding and  
20 good binding affinity. This was done by generating a library of camel  $V_{HH}$   
fragments derived from the dromedary IgG2 and IgG3 isotype, display of  
the  $V_{HH}$  library on phage as fusion proteins with the gene III protein of  
bacteriophage M13 to allow selection of the antigen binders, and finally of  
expressing and extracting the soluble and functional  $V_{HH}$  fragments from  
25 E.coli. As antigen, we choose the tetanus toxoid was chosen because  
comparisons are possible with published data. In addition, the tetanus  
toxoid is a highly immunogenic protein that is routinely used as a vaccine  
in humans to elicit neutralizing antibodies. The two camel  $V_{HH}$  fragments  
that were identified were specific and of high affinity. The affinities of the  
30 two camel  $V_{HH}$  fragments appear to be comparable with those from the  
human anti-tetanus toxoid  $F_{AB}$ 's recently obtained by Mullinas et al. (1990)  
and by Persson et al. (1991).



## 5 Camel immunization

The serum of a camel (*Camelus dromedarius*) was shown to be non-reacting with tetanus toxoid (RIT, Smith Kline Beecham, Rixensart, Belgium). This camel was injected with 100 µgr tetanus toxoid at days 9, 30, 52, 90 and with 50 µgr at days 220, 293 and 449. The blood was  
10 collected 3 days after each injection.

### mRNA purification of camel blood lymphocytes

15 Peripheral blood lymphocytes were purified with Lymphoprep (Nycomed, Pharma) from the bleeding at day 452. Aliquots of  $1 \cdot 10^6$  -  $5 \cdot 10^6$  cells were pelleted and frozen at - 85°C and subsequently used as an enriched source of B-cell mRNA for anti-tetanus toxoid.

20 The mRNA was prepared from a total of  $10^6$  peripheral blood lymphocytes either by the "Micro FastTrack" mRNA isolation kit (Invitrogen) or the "QuickPrep Micro mRNA Purification" kit of Pharmacia, following the recommendations of the manufacturer. With both protocols, up to a few µgr of mRNA was obtained which was used in the subsequent cDNA synthesis  
25 step.

### cDNA synthesis and PCR amplification of camel V<sub>HH</sub> gene

The first-strand cDNA was synthesized with the Invitrogen "cDNA-cycle" or the Pharmacia "Ready-To-Go" kit. The first-strand cDNA was used  
30 immediately afterwards for the specific amplification of the camel V<sub>HH</sub> region by PCR. The primers used have following sequences : the BACK primer (5'-GA TGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'), the internal PstI site is underlined) is designed to hybridize to the framework 1

5 region (codons 1 to 10) of the camel  $V_{HH}$ , while the FOR primer (5'-CGCCATCAAGGTACCAGTTGA-3') hybridizes in the CH2 region. The PCR was carried out with the Taq polymerase from Boehringer Mannheim.

The PCR product was purified according to standard protocols  
10 (Sambrook et al., 1989) and digested with the PstI restriction enzyme of which the target site occurred in the BACK primer, and with BstEII which has a naturally occurring site in the framework 4 of the camel  $V_{HH}$  regions. The resulting fragments of approximately 360 bp (FIGURE 1) were ligated into the pHEN4 vector cut with the same restriction enzymes. The pHEN4  
15 vector (FIGURE 2) is the pHEN1 phasmid (Hoogenboom et al., 1991) - a pUC119 based vector - where the myc-tag was replaced by the decapeptide tag present in the ImmunoZAP H vector (Stratagene). Also the polylinker was modified to allow the cloning of the camel  $V_{HH}$  gene between a PstI and a BstEII site located after the PelB leader signal and in front of  
20 the decapeptide tag and gene III of bacteriophage M13.

### Construction of a camel $V_{HH}$ library

The ligated DNA material was precipitated with 10 volumes and  
25 resuspended in 10  $\mu$ l water and electrotransformed in E.coli XL1 Blue MRF' cells (Stratagene). After electroporation according to the recommended protocol (Stratagene) we kept the cells for 1 hour at 37°C in 1 ml SOC medium before plating on LB plates containing 100  $\mu$ g ampicilline/ml. After an over night incubation at 37°C the transformed cells  
30 were grown out into colonies and some 500,000 recombinant clones were obtained. About 20 colonies, randomly selected, were toothpicked and grown in selective medium (LB/Ampicilline) to prepare plasmid DNA and to check their insert by sequencing. For each clone tested, we found a different  $V_{HH}$  region with the aminoacid sequence and contents

- 5 characteristic for a  $V_{HH}$  originating from a camel heavy chain immunoglobulin (Muyldermans et al., 1994). This indicates that a vast camel  $V_{HH}$  library was generated.

The remaining 500,000 clones were scraped from the plates with a  
10 minimal amount of LB containing 50% glycerol and stored at -85°C until further use.

#### **Panning with tetanus toxoid**

- 15 The library was screened for the presence of anti-tetanus toxoid camel  $V_{HH}$ 's by panning. To this end, approximately  $10^9$  cells (=5 ml suspension of the frozen recombinant clones) were grown to midlogarithmic phase in 200 ml of LB medium supplemented with 1% glucose and 100 µg ampicilline/ml before infection with M13K07  
20 bacteriophages. After adsorption of the bacteriophages on the E.coli cells for 30 min at room temperature, the cells were harvested by centrifugation and washed in LB medium supplemented with ampicilline and kanamycin (25µg/ml). The cells were incubated overnight at 37°C to secrete the recombinant pHEN phasmid packaged within the M13virion containing a  
25 camel  $V_{HH}$  fused to some of its M13gene III proteins (Hoogenboom et al., 1991). The phagemid virions were prepared according to the protocol described by Barbas et al. (1991). The phage pellets were resuspended in blocking solution (1% casein in phosphate buffered saline, PBS), filtered through a 0.2 µm filter into a sterile tube and used for panning. For the  
30 panning the Falcon 3046' plates were coated overnight with 0.25 mg/ml or 2 mg/ml tetanus toxoid dissolved in PBS or hydrogencarbonate pH 9.6. The wells were subsequently washed and residual protein binding sites were blocked with blocking solution at room temperature for 2 hours. The adsorption of the phagemid virions on the immobilized antigen and the

5 washing and elution conditions were according to Marks et al (1991) or were taken from the protocol described by the «Recombinant Phage Antibody System » of Pharmacia 4 consecutive rounds of panning were performed. After the fourth round of panning the eluted phagemid virions were added to exponentially growing TGI cells (Hoogenboom et al. 1991)  
10 and plated on ampicilline containing LB plates. After overnight growth several colonies were grown individually in LB medium to midlogarithmic growing phase, and infected with M13K07 helper phage. The virions were prepared and tested for their binding activity against tetanus toxoid immobilised on microtiter plates. The presence of the virion binding to the  
15 immobilized antigen was revealed by ELISA using a Horse Radish Peroxidase/anti-M13 conjugate (Pharmacia). The percentage of binders was increasing after each round of panning. In the original library we found 3 clones out of 96 which showed binding with the immobilized tetanus toxoid. This number was increased to 11, 48 and 80 after the first,  
20 second and third round of panning. All of the individual clones which were tested after the fourth round of panning were capable to recognize the antigen, as measured by ELISA (FIGURE 3). Ten positive clones were grown and tested by PCR to check the presence of an insert with the proper size of the  $V_{HH}$  gene, and their DNA was finally sequenced. The  
25 sequencing data revealed that two different clones were present among this set of 10 clones. The phasmid DNA of these clones was named pHEN4- $\alpha$ TT1 and pHEN4- $\alpha$ TT2, (The pHEN4- $\alpha$ TT2 phasmid DNA was deposited at the "Belgian Coordinated Collections of Microorganisms" BCCM/LMBP on January 31, 1995 under accession number LMBP3247),  
30 and it was shown that these two different clones contained a cDNA coding for a camel  $V_{HH}$  (FIGURE 4). Comparison of the amino acids in these clones with the camel  $V_{HH}$  clones analysed before (Muyldermans et al., 1994) clearly indicated that the anti-tetanus camel  $V_{HH}$  originated from a heavy chain immunoglobulin lack the CH1 domain and light chains.

- 5 Especially the identity of the key residues at position 11 (Ser), 37 (Phe) and 45 (Arg or Cys) and 47 (Leu or Gly) proved this statement (Muyldermans et al., 1994).

**Production of soluble camel V<sub>HH</sub> with anti-tetanus toxoid activity**

10

The phasmid DNA of the two clones which scored positive in the tetanus toxoid ELISA were transformed into WK6 cells. These cells are unable to suppress the stopcodon present in the vector between the decapeptide tag and the gene III protein. The WK6 *E.coli* cells harboring the pHEN4-aTT1  
15 or pHEN4-aTT2 phasmid were grown at 37°C in 1 liter of TB medium with 100 mgr ampicillin/ml and 0.1% glucose. When the cells reached an OD<sub>550</sub> of 1.0 we harvested the cells by centrifugation at 5000 rpm, 10 minutes. The cell pellet was washed once in TB medium with ampicillin, but ommitting the glucose. The cells were finally resuspended in 1 liter of  
20 TB medium with ampicillin (100 µgr/ml). We induced the expression of the camel V<sub>HH</sub> domain by the addition of 1 mM IPTG and further growth of the cells at 28°C for 16 hours. The expressed proteins were extracted from the periplasmic space following the protocol described by Skerra and Pluchthun (1988). We pelleted the *E.coli* cells by centrifugation at 4000g for 10 min.  
25 (4°C). The cells were resuspended in 10 ml TES buffer (0.2 M Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5 M sucrose). The suspension was kept on ice for 2 hours. The periplasmic proteins were removed by osmotic shock by addition of 20 ml TES diluted 1/4 with water. The suspension was kept on ice for 1 hour and subsequently centrifuged at 12,000 g for 30 minutes at  
30 4°C. The supernatant contained the expressed camel V<sub>HH</sub> domain. The extract corresponding to 400 µl cell culture was applied under reducing conditions on a SDS/polyacrylamide protein gel. The extracted proteins were visualized in the SDS/polyacrylamide gels by Coomassie blue staining (FIGURE 5). A protein band with an apparent molecular weight of

5 16,000 D was clearly present in the E.coli cultures containing the recombinant clones and induced with IPTG. Alternatively, the presence of the camel  $V_{HH}$  proteins in the extract was revealed with IPTG. Alternatively, the presence of the camel  $V_{HH}$  proteins in the extract was revealed by Western blot using a specific rabbit anti-camel  $V_{HH}$  or rabbit  
10 anti-dromedary IgG serum or the anti-tag antibody.

We estimate from the band intensity observed in the Coomassie stained gel that more than 10 mg of the camel  $V_{HH}$  protein (non-purified) can be extracted from the periplasm of 1 liter induced *E.coli* cells.

15 For the purification of the anti-tetanus toxoid camel  $V_{HH}$  we concentrated the periplasmic extract 10 times by ultrafiltration (*Milipore* membrane with a cut off of 5000 Da). After filtration the concentrated extract from the pHEN4- $\alpha$ TT2 was separated according its molecular weight by gelfiltration on Superdex-75 (*Pharmacia*) (FIGURE 6)  
20 equilibrated with PBS (10 mM phosphate buffer pH7.2, 150 mM NaCl). The peak containing the anti-tetanus toxoid activity eluted at the expected molecular weight of 16,000 Da indicating that the protein behaved as a monomer and doesn't dimerize in solution. The fractions containing the pure  $V_{HH}$  (as determined by SDS-PAGE) were pooled and the  
25 concentration was measured spectrophotometrically using a calculated  $E_{280}$  (0.1%) of 1.2 and 2.3 respectively for the  $\alpha$ TT1 and  $\alpha$ TT2. From the UV absorption at 280 nm of the pooled fraction we could calculate a yield of 6 mgr of purified protein per liter of bacterial culture. The purified protein could be further concentrated by ultrafiltration to 6 mgr/ml in PBS or  
30 water without any sign of aggregation, as seen on the UV spectrum.

Concerning the expression yield in *E.coli* it should be realized that at this stage we didn't try to optimize the expression or the protein extraction conditions. However, as the yield of the purified  $\alpha$ TT2 camel  $V_{HH}$  reached

5 6 mgr per liter of bacterial culture, and as we obtained the soluble protein at a concentration of 6 mgr/ml, it is clear that the expression is comparable or better than other scFv's or F<sub>AB</sub>'s expressed in *E.coli*. Furthermore, the solubility of the camel V<sub>HH</sub> aTT2 is certainly better than that obtained for the mouse VH fragments. The yield and solubility is certainly in the range  
10 needed for most applications.

To prove the proper folding of the purified protein, the αTT2 was brought at a concentration of  $3.9 \times 10^{-6}$  M and used it for CD measurement (FIGURE 7). The CD spectrum is characteristic for a polypeptide with a β-pleated sheet folding as expected for a well structured immunoglobulin  
15 fold (Johnson, 1990).

#### **The camel anti-tetanus toxoid V<sub>HH</sub> affinity measurements**

20 The binding of the camel V<sub>HH</sub> antibody to the tetanus toxoid immobilised on the microtiter plates was revealed by the successive incubation with firstly, the rabbit anti-camel V<sub>HH</sub> or rabbit anti-dromedary IgG and secondly a goat anti-rabbit/alkaline phosphatase conjugated antibodies (Sigma). The apparent affinity of the camel V<sub>HH</sub> proteins against  
25 tetanus toxoid was estimated by inhibition ELISA exactly as described by Persson et al. (1991) for the human anti-tetanus toxoid F<sub>AB</sub> fragments they produced in E.coli

The specificity of the soluble camel V<sub>HH</sub> for the tetanus toxoid was  
30 suggested from the ELISA experiments in which we competed the binding with free antigen was competed. An apparent inhibition constant of around  $10^{-7}, 10^{-8}$  M was observed for both V<sub>HH</sub> fragments (FIGURE 8). This compares favorable with the inhibition constants for the human anti-tetanus

- 5     toxoid F<sub>AB</sub> fragments cloned by Persson et al. (1991) which were in the range of  $10^{-7}$  to  $10^{-9}$  M.

          The measurement of the affinity constant by ELISA is however, more reliable if determined according to the procedure of Friguet *et al.* (1987).  
10    With this protocol we found an affinity constant of  $6 \cdot 10^7$  M<sup>-1</sup> and  $2 \cdot 10^7$  M<sup>-1</sup> for the  $\alpha$ TT1 and  $\alpha$ TT2 respectively. These affinities are consistent with a specific V<sub>HH</sub>-antigen interaction (the polyspecific antibodies generally bind their antigen with affinities of  $10^6$  M<sup>-1</sup> or less (Casali *et al.* 1989)).

15    **Epitope recognition of  $\alpha$ TT1 and  $\alpha$ TT2.**

          Tetanus toxin consists of three domains. The C fragment binds to the neuronal cells, it is said to be the neurospecific binding domain. The B domain appears to be involved in the neuronal penetration of the A domain or L chain (Montecucco & Schiavo, 1993). The L chain is responsible for  
20    the intracellular activity.

          The C fragment is the most immunogenic part of the tetanus neurotoxin, and a recombinant C fragment is commercially available (*Boehringer and Calbiochem*). We showed by ELISA that the  $\alpha$ TT1 bacterial extract binds equally well both to the complete tetanus toxoid and  
25    to the recombinant C fragment. Therefore the epitope of this camel V<sub>HH</sub> is present on the C fragment. By contrast, the  $\alpha$ TT2 extract binds to the complete tetanus toxoid, but not to the C fragment. Therefore the  $\alpha$ TT2 recognizes an epitope located on the A or B domain.

30    **The *in vivo* neutralization of tetanus toxin toxicity.**

          The neutralizing activity of the purified camel  $\alpha$ TT1 or  $\alpha$ TT2 V<sub>HH</sub> domains against tetanus toxin was tested. As a control, eight NMRI mice of 8 to 12 weeks (80 to 100 gr) were injected I.P. with 400 ngr tetanus toxin



5 (SmithKline Beecham Biologicals) (= 10 times the LD50) in 0.1 ml PBS. To test the neutralizing activity of the camel  $V_{HH}$   $\alpha$ TT1 or  $\alpha$ TT2 we preincubated 4 or 40 mgr of this purified recombinant protein with 400 ngr of the tetanus toxin in 0.1 ml of PBS for 30 minutes before I.P. injection into the mice. The survival of the mice was followed over a period of 2 weeks  
10 (FIGURE 9). It is clear that all mice injected with the tetanus toxin alone or in the presence of a non-specific purified camel  $V_{HH}$  (cVH21 of Muyldermans *et al.*, 1994) were killed within 3 days. The survival of the mice injected with the tetanus toxin was increased significantly by the co-injection of only 4 mgr of the purified camel  $\alpha$ TT1 or  $\alpha$ TT2. The survival  
15 was even more pronounced for the co-injection of tetanus toxin with 40 mgr of camel  $V_{HH}$ . It appears that the  $\alpha$ TT1 had a slightly higher neutralizing activity than the  $\alpha$ TT2. This could originate from its intrinsic higher affinity for binding the tetanus toxin (Simpson *et al.*, 1990). Alternatively it might result from the binding of the  $\alpha$ TT1  $V_{HH}$  to the fragment C of the tetanus  
20 toxin which inhibits more the toxic effect than the binding of the  $\alpha$ TT2 to its epitope outside the C fragment.

## **EXAMPLE II : GENERATION OF SPECIFIC CAMEL $V_{HH}$ FRAGMENTS**

### **25 AGAINST LYSOZYME**

Using the same protocol as the one described in Example I (specific steps or conditions modifying those of example I are indicated hereafter) for the generation of specific camel  $V_{HH}$  fragments having a specificity and an affinity for tetanus toxoid,  $V_{HH}$  fragments have been obtained against  
30 lysozyme.

We choosed the Hen Egg Lysozyme (HEL) as an antigen to immunize a camel (*Camelus dromedarius*). This protein was selected for the reason that comparisons can be made with several other mouse

- 5 monoclonal antibody fragments recognizing the same antigen and of which the structure even in complex with its antigen are known.

### **Camel immunization**

- 10 The serum of a camel was shown to be non-reacting with lysosyme. We injected this camel with 100 µg lysozyme (Boehringer) at days 9, 30, 52, 90 and with 50 µg at days 220, 293 and 449. The blood was collected on average 3 days after each injection.

The following steps were then performed as in Example I.

- 15
- mRNA purification of camel blood lymphocytes.
  - cDNA synthesis and PCR amplification of camel V<sub>HH</sub> gene.
  - Construction of Camel V<sub>HH</sub> library.
  - Panning with lysozyme (the Falcon 3046' plates were coated with 1 mg/ml lysozyme).

- 20 96 colonies were randomly chosen and grown individually in LB medium.

The virions were prepared and tested for their binding activity against lysozyme immobilised on microtiter plates.

- 25 The percentage of binders was increasing after each round of panning. Twenty positive clones were grown and tested by PCR to check the presence of an insert with the proper size of the V<sub>HH</sub> gene, and their DNA was finally sequenced. The sequencing data revealed that two different clones were present among this set of 10 clones. The phasmid DNA of these clones was named pHEN4-αLYS2 and pHEN4-αLYS3, and it
- 30 was shown that these two different clones contained a cDNA coding for a camel V<sub>HH</sub> (Figures 11, 12). Comparison of the amino acids in these clones with the camel V<sub>HH</sub> clones we analysed before (Muyldermans et al., 1994) clearly indicated that the anti-lysozyme camel V<sub>HH</sub> originated from a heavy chain immunoglobulin lacking the CH1 domain and light chains. Especially

5 the identity of the key residues at position 11 (Ser), 37 (Phe), 44 (Glu), 45 (Arg) and 47 (Gly) proved this statement (Muyldermans et al., 1994).

- Production of soluble camel V<sub>HH</sub> with anti-lysozyme activity.

For the purification of the anti-lysozyme camel V<sub>HH</sub> we concentrated the periplasmic extract 10 times by ultrafiltration (Milipore membrane with a cut off of 5000 Da). After filtration the concentrated extract from the PHEN4- $\alpha$ LYS2 can be purified by Protein A-Sepharose chromatography. Elution of the  $\alpha$ LYS2 V<sub>HH</sub> is done with 100 mM Tri-ethanol amine. The pH of eluate is immediately neutralized with 1 M Tris-HCl (pH 7.4). Unfortunately the expressed  $\alpha$ -LYS3 V<sub>HH</sub> does not bind to Protein A.  
15 Therefore the purification has to be performed by affinity chromatography. The concentrated extract is applied on a column of lysozyme immobilized on CNBr-Sepharose (Pharmacia). Elution of the anti-lysozyme V<sub>HH</sub> is obtained with 100 mM Tri-ethanolamine. The eluate has to be neutralized as described above. Further purification of both anti-lysozyme V<sub>HH</sub>'s can  
20 obtained by gel filtration on Superdex-75 (Pharmacia) equilibrated with PBS (10 mM phosphate buffer pH7.2, 150 mM NaCl). The peak containing the anti-lysozyme activity eluted at the expected molecular weight of 16,000 Da indicating that the protein behaved as a monomer and doesn't dimerize in solution. The fractions containing the pure V<sub>HH</sub> (as determined by SDS-  
25 PAGE) were pooled and the concentration was measured spectrophotometrically. A yield of 5 mg of purified protein per liter of bacterial culture was calculated. The purified protein could be further concentrated by ultrafiltration to 10 mg/ml in PBS or water without any sign of aggregation, as seen on the UV spectrum.

- 30 • The camel anti-lysozyme V<sub>HH</sub> affinity measurements

The specificity of the soluble camel V<sub>HH</sub> for the lysozyme was suggested from the ELISA experiments in which we competed the binding with free antigen. An apparent inhibition constant of around  $5 \cdot 10^{-7}$  and  $5 \cdot 10^{-8}$  M was observed for the  $\alpha$ -LYS3 and  $\alpha$ -LYS2 respectively. These

5 affinities are consistent with a specific  $V_{HH}$ -antigen interaction (the polyspecific antibodies generally bind their antigen with affinities of  $10^6 M^{-1}$  or less (Casali et al. 1989).

- Epitope recognition of  $\alpha$ -LYS2 and  $\alpha$ -LYS3.

10 To analyse whether the two camel  $V_{HH}$  with anti-lysozyme activity bind to the same or to different epitopes we used the techniques of additive binding in ELISA (Friguet et al., 1989). An additivity index of more than 40 indicates pairs of antibodies that can bind simultaneously on the antigen, while additivity indices of less than 20 is characteristic for pairs of antibodies  
15 with overlapping epitopes. Our camel  $\alpha$ -LYS2 and  $\alpha$ -LYS3 had an additivity index of 45. From this experiment it appears that the  $\alpha$ -LYS2 and  $\alpha$ -LYS3 bind to different epitopes on the lysozyme molecule.

### **EXAMPLE 3 : MAKING BIVALENT MONOSPECIFIC OR MONOVALENT** 20 **BISPECIFIC BINDING CONSTRUCTS FROM CAMELID $V_{HH}$ 'S**

From the camel  $V_{HH}$ 's with specificity to tetanus toxin ( $\alpha$ -TT1 or  $\alpha$ -TT2) or with specificity to lysozyme ( $\alpha$ -LYS2 or  $\alpha$ -LYS3) cloned in the pHEN-4 bacterial expression vector, we made constructs with following characteristics:

- 25 1.  $V_{HH}$  with ProX repeat sequences of the camel long hinge including the 3 Cys and part of the CH2 domain. These constructs can be also used as an intermediate for the next constructs.
2.  $V_{HH}$  with ProX repeat sequences of the long hinge of camel with one Cys followed by a stopcodon in the pHEN4. These are bivalent  
30 constructs with monospecificity.
3.  $V_{HH}$  linked with the ProX repeat sequences of the long hinge of camel (without Cys) followed by a second  $V_{HH}$ . These are monovalent constructs with bispecificity, or bivalent constructs with monospecificity depending on the  $V_{HH}$ 's.

5

1. **Camel V<sub>HH</sub> with camel long hinge and part of CH2 domain.**

The (pHEN4- $\alpha$ -LYS3) or the (pHEN4- $\alpha$ -TT2) plasmids were digested  
10 with BstEII and Xmn I. BstEII cuts in the framework 4 of the camel V<sub>HH</sub>, and  
Xmn I cuts in the  $\beta$ -lactamase gene of pHEN4. The DNA fragment  
containing the camel V<sub>HH</sub> was isolated from agarose gel.

A clone containing a camel V<sub>HH</sub> with unknown specificity, the camel  
long hinge and the first part of the CH2 domain cloned in pBluescript  
15 (Statagene) was cut with the same enzymes (Bst EII and Xmn I) and the  
DNA resulting fragment containing the hinge and CH2 parts was isolated  
from agarose gel.

The two DNA fragments (one containing the V<sub>HH</sub> of determined  
specificity, the other containing the coding sequence of the hinge and CH<sub>2</sub>  
20 domains) were mixed and ligated to each other and used to transform E.  
coli cells. As a result a (pHEN4- $\alpha$ -LYS3-long hinge-CH2) plasmid and a  
(pHEN4- $\alpha$ -TT2-long hinge-CH2) plasmid have been obtained.

2. **Bivalent monospecific constructs (Figures 13, 15).**

25 The (pHEN4- $\alpha$ -LYS3-long hinge-CH2) plasmid was taken as  
template for amplification with primers A4 and AM007.

**A4** (Sf I site underlined):

5'CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCGA(G,T)GT(G,C)C  
AGCT-3'

30

**AM007:**

5'GGCCATTTGCGGCCGCATTCCATGGGTTTCAGGTTTTGG-3'

5 These primers anneal respectively with their 3' end to the beginning of the  $V_{HH}$  and to the end of the structural upper hinge of the camel long hinge sequence. The primer AM007 will extend the 3' end of the  $\alpha$ -LYS3 or of the  $\alpha$ -TT2 gene (depending on the template) with CCCATGGAATGCGGCCGCAAATGTCC. The NcoI and NotI sites are  
10 underlined. These nucleotides up to the Not I site code for the amino acids Pro Met Glu Cys.

The PCR fragment is double digested with Sfi I and Not I, and the resulting fragments are cloned in the pHEN-4 vector cleaved with the same enzymes. The ligated material is transformed in WK6 E. coli cells and  
15 selected on ampicillin. The transformed clones are checked for their insert by PCR and by sequencing. The plasmid (pHEN4- $\alpha$ -LYS3-long hinge/Cys) and (pHEN4- $\alpha$ -TT2-long hinge/Cys) were generated.

The extraction of the expressed  $V_{HH}$   $\alpha$ -LYS3-long hinge/Cys or  $\alpha$ -TT2-long hinge/Cys proteins lead to isolation of a dimerised molecule  
20 because of the formation of the disulfide bridge between the Cys residue within the long hinge. Both camel  $V_{HH}$  dimer constructs ( $\alpha$ -LYS3 long hinge/Cys)<sub>2</sub> and  $\alpha$ -TT2 long hinge/Cys)<sub>2</sub> are well expressed in E. coli upon induction with IPTG, and are easily obtained from the periplasm. They were quite soluble and bound the original antigen with high affinity and  
25 high specificity.

### 3. Monovalent bispecific protein constructs (Figures 14, 16).

In the previous plasmid constructs (pHEN4- $\alpha$ -LYS3-long hinge/Cys)  
30 and (pHEN4- $\alpha$ -TT2-long hinge/Cys), we have two restriction sites for Nco I. Digestion of the plasmid with this enzyme allows the isolation of the camel  $V_{HH}$  gene followed by the long hinge without the Cys codon. Ligation of the ( $\alpha$ -LYS3-long hinge) fragment into the pHEN4- $\alpha$ -LYS2 or in the pHEN4- $\alpha$ -TT2 plasmids linearised with NcoI creates the plasmids (pHEN4- $\alpha$ -LYS3-

5 long hinge linker- $\alpha$ -LYS2) or (pHEN4- $\alpha$ -LYS3-long hinge linker- $\alpha$ -TT2). Expression of the gene leads to the production of the  $\alpha$ -LYS3  $V_{HH}$  linked to the  $\alpha$ -LYS2  $V_{HH}$  or linked to the  $\alpha$ -TT2  $V_{HH}$  by the intermediate of a linker based on the structural upper hinge of the camel long hinge.

10 Following this protocol monovalent bispecific proteins consisting of the camel  $V_{HH}$  of  $\alpha$ -LYS3 linked to the camel  $V_{HH}$  of  $\alpha$ -LYS2 and the that of camel  $V_{HH}$  of  $\alpha$ -LYS3 linked to the camel  $V_{HH}$  of  $\alpha$ -TT2 can be isolated. Both proteins are expressed well in E. coli and can be extracted from the periplasm. In ELISA the binding properties of the latter protein to the  
15 tetanus toxoid and to the lysosyme can be shown.

With these gene constructs at hand it becomes possible and straightforward to exchange either  $V_{HH}$  with any other  $V_{HH}$  with another specificity.

- For example we can exchange the second camel  $V_{HH}$  by digesting the  
20 plasmid with Pst I, or with Nco I and to ligate the DNA fragment containing the  $V_{HH}$ -long hinge linker into the pHEN4- $V_{HH}$  linearised with either Pst I or Nco I.
- Similarly, we exchanged the first camel  $V_{HH}$   $\alpha$ -LYS3 gene from the (pHEN4- $\alpha$ -LYS3-long hinge linker- $\alpha$ -LYS2) plasmid construct into  
25 (pHEN4- $\alpha$ -TT1-long hinge linker- $\alpha$ -LYS2). This was done by cutting the plasmid with Bst EII and further ligating the DNA fragment containing the (long hinge linker- $\alpha$ -LYS2) into the (pHEN4- $\alpha$ -TT1) plasmid linearised with Bst EII.
- With a slight modification of this protocol it becomes even possible to  
30 generate multivalent constructs. In this case the ( $V_{HH}$ -long hinge linker- $V_{HH}$ ) plasmid needs to be digested with Bst EII and the DNA fragment containing the (long hinge linker- $V_{HH}$ ) gene should be isolated from agarose gel. Because of the asymmetry in the recognition site of Bst EII, it is only possible to obtain head-to tail ligations upon self ligation. The

- 5 self-ligated DNA should thereafter (with or without prior size selection) be ligated into the pHEN4-V<sub>HH</sub> plasmid linearised with Bst EII. This will create a plasmid of the type (pHEN4-[V<sub>HH</sub>-long hinge linker]<sub>n</sub>).



5

**REFERENCES** cited in the preceding examples

- Borrebaeck *et al.*, (1992) *Bio/Technology* 10, 697-698.
- Casali *et al.*, (1989) *Annu. Rev. Immunol.* 7, 513-536.
- Friguet *et al.*, (1983) *J. Immunol. Meth.* 60, 351 et (1989) *Protein Structure.*
- 10 A practical approach (Ed. T.E. Creighton) IRL Press p. 287-310)
- Glockshuber *et al.*, (1990) *Biochemistry* 29, 1362-1367.
- Hamers-Casterman *et al.*, (1993) *Nature* 363, 446-448.
- Hoogenboom *et al.*, (1991) *Nucl. Acids Res.* 19, 4133-4137.
- Johnson W.C., (1990) *Proteins: Structure, Func & Genetics* 7, 205-214.
- 15 Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest* (US Dept. Health Human Services, Washington) 5th Ed.
- Marks *et al.*, (1991) *J. Mol. Biol.* 222, 581-597.
- Montecucco & Schiavo (1993) *TIBS* 18, 324-329.
- Mullinax *et al.*, (1990) *Proc. Natl. Acad. Sci USA* 87, 8095-8099.
- 20 Persson *et al.*, (1991) *Proc. Natl. Acad. Sci USA* 88, 2432-2436.
- Sambrook *et al.*, (1989) *Molecular Cloning* CSHL Press
- Simpson *et al.*, (1990) *J. Pharmacol. & Exp. Therap.* 254, 98-103.
- Skerra and Pluckthun, (1988) *Science* 240, 1038-1040.

5

### CLAIMS

1. Variable fragment ( $V_{HH}$ ) of a heavy chain of an immunoglobulin devoid of light chains, which is encoded by a nucleotide sequence obtainable by the following process:
- 10 - treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,
- 15 - contacting the obtained cDNA with at least two different primer oligonucleotides in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a
- 20 FOR primer (forp 1) replying to the following nucleotide sequence 5'-CGCCATCAAGGTACCGTTGA-3' or 5'-CGCCATCAAGGTACCAAGTTGA-3'
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- 25 - recovering amplified DNA corresponding to bands of different size orders including:
- . a band of around 750 basepairs which is the amplified product of the variable heavy chain ( $V_H$ ), CH1, hinge and part of CH2 region of a four-chain immunoglobulin,
  - 30 . a band of around 620 basepairs which is the amplified product of the variable heavy-chain ( $V_{HH}$ ), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,
  - . a band of around 550 basepairs which is the amplified product of the variable heavy-chain ( $V_{HH}$ ), short hinge, and part of the CH2 of the camel
  - 35 two-chain immunoglobulin IgG3,

- 5 - purifying the two shortest bands from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the  $V_{HH}$  fragments,
- digesting the amplified products with restriction enzymes having target
- 10 sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified
- 15 fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- 20 - infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- incubating the recombinant host cells in conditions allowing secretion of recombinant phagemid virions particles containing the recombinant
- 25 phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
- submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the adsorption of the phagemid virions on the immobilized antigen,
- 30 - eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,
- recovering the virions and testing them for their binding activity against
- 35 the antigen of interest, for example by ELISA,

- 5    - recovering the phagemid virions having the appropriate binding activity,  
- isolating the nucleotide sequence contained in the phasmid vector and  
capable of being expressed on the phagemid virions as a  $V_{HH}$  aminoacid  
sequence having the appropriate binding activity.

2.    Variable fragment ( $V_{HH}$ ) of a heavy chain of an  
10 immunoglobulin, which is encoded by a nucleotide sequence obtainable by  
a process according to the one disclosed in claim 1, wherein a  
reamplification step of the 620 basepairs and 550 basepairs PCR product  
of claim 1 performed with oligonucleotide primers having respectively the  
following nucleotide sequences:

15    BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

FOR primer: 5'- CG ACT AGT GCG GCC GCG TGA GGA GAC GGT GAC  
CTG-3'

3.    Variable fragment ( $V_{HH}$ ) of a heavy chain of an immunoglobulin,  
20 which is encoded by a nucleotide sequence obtainable by a process  
according to the one disclosed in claim 1, wherein the amplification step of  
the cDNA obtained from the mRNA is performed with oligonucleotide  
primers having respectively the following nucleotide sequences:

BACK primer: 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3'

25

FOR primer 3: 5'- TGT CTT GGG TTC TGA GGA GAC GGT -3'

FOR primer 4: 5'- TTC ATT CGT TCC TGA GGA GAC GGT -3'

4.    Variable fragment of a heavy chain of an immunoglobulin devoid of  
30 light chains according to anyone of claims 1 or 2, encoded by a nucleotide  
sequence obtainable from blood lymphocytes or other appropriate cells of  
camelids wherein the camelids have been immunized with a determined  
antigen prior to the treatment of their blood lymphocytes or other  
appropriate cells.

- 5 5. Variable fragment of a heavy chain of an immuglobulin according to anyone of claim 1 to 4 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids characterized in that the camelids have been previously immunized with an antigen which is a toxin of a bacteria or the corresponding toxoid.
- 10 6. Variable fragment of a heavy chain of an immuglobulin according to claim 5 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids wherein the antigen is the tetanus toxoid of Clostridium tetani.
7. Variable fragment of a heavy chain of an immuglobulin encoded by  
15 a nucleotide sequence according to claim 5 wherein the antigen is a bacterial toxin or toxoid chosen among those of the following bacteria: Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxig E. Coli, Salmonella,  
20 Shigella, Listeria.
8. Variable fragment of a high chain of an immuglobulin according to anyone of claims 1 to 4 encoded by a nucleotide sequence obtainable from blood lymphocytes or other appropriate cells of camelids, wherein the camelids have been immunized with an antigen present in venom of  
25 animals.
9. Variable fragment of a high chain of an immuglobulin according to claim 8, encoded by a nucleotide sequence wherein the antigen is a toxin or toxoid chosen among those produced by anemonies, coral, jellyfish, spiders, bees, wasps, scorpions, snakes, including those belonging to the  
30 families of Viperidae, Crotalidae, Lapidea.
10. Variable fragment of a heavy chain immunoglobulin encoded by a nucleotide sequence obtained by a process of anyone of claims 1 to 9.
11. Variable fragment of a heavy chain of an immuglobulin, characterized in that it comprises the following aminoacid sequence:

5 (Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu  
 ArgLeuSerCysAla(Ala/Thr)SerGly(CDR1)Trp(Phe/Tyr)ArgGlnAlaProGlyLy  
 s  
 10 Glu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)(CDR2)Arg(Phe/Leu)ThrIleSer(Arg/  
 Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)TyrLeu(Gln/Leu)MetAsnSerLeu  
 (Lys/Glu)ProGluAspThrAla(Val/Met/Ile)TyrTyrCysAlaAla(CDR3)TrpGlyGln  
 15 GlyThrGlnValThrValSerSer or  
 (Glu/Asp)ValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGly(Gly/Gln)SerLeu  
 20 ArgLeuSerCysAla(Ala/Thr)SerGly(Ala, Thr, Ser, Ser/Tyr, Thr, Ile, Gly)(CDR1)  
 Trp(Phe/Tyr)ArgGlnAlaProGlyLysGlu(Arg/Cys)Glu(Gly/Leu)Val(Ser/Ala)  
 (CDR2)Arg(Phe/Leu)ThrIleSer(Arg/Leu/Gln)AspAsnAlaLysAsnThr(Val/Leu)  
 25 TyrLeu(Gln/Leu)MetAsnSerLeu(Lys/Glu)ProGluAspThrAla(Val/Met/Ile)Tyr  
 TyrCysAlaAla(CDR3)TrpGlyGlnGlyThrGlnValThrValSerSer,

30 wherein CDR1, CDR2 and CDR3 represent variable amino acid  
 sequences providing for the recognition of a determined epitope of  
 the antigen used for the immunization of Camelids, CDR1, CDR2  
 and CDR3 sequences comprising from 5 to 25 amino acid residues  
 preferably CDR1 contains from 7 to 12 amino acid residues, CDR2

5 contains from 16 to 21 amino acid residues and CDR3 contains from 7 to 25 amino acid residues.

12. Amino-acid sequence encoded by a nucleotide sequence comprising the sequence starting at nucleotide 400 and ending between nucleotides 479 and 495 of the nucleotides 479 and 495 of the nucleotide  
10 sequence presented on figure 15.

13. Variable fragment of a heavy chain of an immunoglobulin coded by a nucleotide sequence present in recombinant phasmid pHEN4- $\alpha$ TT2(WK6) deposited at the BCCM/LMBP under accession number LMBP3247.

14. Variable fragment of a heavy chain of an immunoglobulin  
15 characterized in that it comprises or it replies to the following  $\alpha$ TT1 sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly**Ser**ValGlnAlaGlyGlySerLeuArgLeu

20 SerCysAlaAlaSerGlyGlyGlnThrPhe**AspSerTyrAlaMetAla**Trp**Phe**ArgGln

AlaProGlyLysGluCysGluLeuValSer**SerIle**lleGly**AspAspAsnArgAsnTyr**

**AlaAspSerValLysGly**ArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr

25

LeuGlnMetAspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln**LeuGly**

**SerAlaArgSerAlaMetTyrCys**AlaGlyGlnGlyThrGlnValThrValSerSer.

15. Variable fragment of a heavy chain of an immunoglobulin  
30 characterized in that it comprises or it replies to the following  $\alpha$ TT2 aminoacid sequence:

GluValGlnLeuGlnAlaSerGlyGlyGly**Ser**ValGlnAlaGlyGlySerLeuArgLeu

5 SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

ProGlyLysGlu**Arg**GluGlyValAla**GlylleSerSerGlyGlySerThrThrAlaTyr**

**SerAspSerValLysGly**ArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

10

LeuLeulleAspAsnLeuGlnProGluAspThrAlalleTyrTyrCysAlaGly**ValSer**

**GlyTrpArgGlyArgGlnTrpLeuLeuLeuAla**GluThrTyrArgPheTrpGlyGlnGly

15 ThrGlnValThrValSerSer.

16. Variable fragment of a heavy chain of an immunoglobulin according to anyone of claims 1 to 15 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of light chains according to anyone of claims 1 to 15, the V<sub>HH</sub> fragments  
20 having the same antigen specificity.

17. Bivalent monospecific construct of variable fragments of an immunoglobulin according to claim 16, which is a dimer of V<sub>HH</sub> fragments having the same specificity.

18. Bivalent monospecific construct according to claim 17, wherein the  
25 V<sub>HH</sub> fragments are linked to each other with the hinge amino-acid sequence of the hinge domain of an immunoglobulin devoid of light chain.

19. Variable fragment of a heavy chains of an immunoglobulin according to anyone of claims 1 to 15 characterized in that it is linked to at least one further variable fragment of heavy chains of an immunoglobulin devoid of  
30 light according to anyone of claims 1 to 15, the V<sub>HH</sub> fragments having different antigen specificities.

20. Multivalent multispecific construct wherein variable fragments according to claim 19 are linked to each other with part of the amino-acid sequence of the hinge domain of an immunoglobulin devoid of light chains,



5 this part being devoid of at least the codon encoding a cysteine residue at the end of the hinge domain.

21. Multivalent multispecific construct according to claim 20, which contains at least two V<sub>HH</sub> fragments having a different antigen- and/or epitope- specificity.

10 22. Construct according to anyone of claims 16 to 18 wherein the sequence of the the hinge domain includes or corresponds to the amino-acid sequence starting at position 400 and ending between position 489 and position 495 as indicated on the sequence of figure 15.

23. Construct according to anyone of claims 19 to 22 wherein the  
15 sequence of part of the hinge region includes or corresponds to the amino-acid sequence starting at position 400 and ending between position 479 and position 486 as indicated on the sequence of figure 15.

24. Pharmaceutical composition, characterized in that it comprises an immunoglobulin variable fragment according to anyone of claims 1 to 6 or  
20 19 or a construct according to anyone of claims 17, 18, 20 to 23 in admixture with a physiologically acceptable vehicle and/or adjuvant(s).

25. Pharmaceutical composition according to claim 16 for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium  
25 Perfringens, Staphylococcus, Pseudomonas, Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially Vibrio cholera, enterotoxigenic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, bees, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.

30 26. immunoglobulin variable fragment according to anyone of claims 2 to 6 or 19, or a construct according to anyone of claims 17, 18, 20 to 23 for use for the treatment by passive immunisation, of infection or acute intoxication by toxins such as those of Clostridium, especially Clostridium Botulinum or Clostridium Perfringens, Staphylococcus, Pseudomonas,  
35 Pasteurella, Yersinia, Bacillus Anthracis, Neisseria, Vibrio, especially

- 5 Vibrio cholera, enterotoxic E. Coli, Salmonella, Shigella, Listeria or anemonies, coral, jellyfish, spiders, bees, wasps, scorpions, snakes, including those belonging to the families of Viperidae, Crotalidae, Lapidea.
27. Nucleotide sequence coding for a variable fragment ( $V_{HH}$ ) of a heavy chain of an immunoglobulin obtainable by the following process:
- 10 - treating blood lymphocytes or other appropriate cells of an animal of the Camelid family previously immunized with a determined antigen, in order to give access to their mRNA,
- synthesizing a first strand of cDNA starting from the obtained mRNA,
- contacting the obtained cDNA with at least two different oligonucleotide
- 15 primers in conditions allowing their hybridization to at least two complementary nucleotide sequences contained in the cDNA, said primers comprising a BACK primer (back p1) having the following nucleotide sequence 5'-GATGTGCAGCTGCAGGCGTCTGG(A/G)GGAGG-3' and a FOR primer (forp 1) replying to the following nucleotide sequence
- 20 5'-CGCCATCAAGGTACCGTTGA-3' or 5'-CGCCATCAAGGTACCAAGTTGA-3'
- amplifying the DNA fragment located between the nucleotide sequence hybridized with said primers and,
- recovering amplified DNA corresponding to bands of different size orders
- 25 including:
- . a band of around 750 basepairs which is the amplified product of the variable heavy chain ( $V_H$ ), CH1, hinge and part of CH2 region of a four-chain immunoglobulin,
  - . a band of around 620 basepairs which is the amplified product of the

30 variable heavy-chain ( $V_{HH}$ ), long hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG2,

  - . a band of around 550 basepairs which is the amplified product of the variable heavy-chain ( $V_{HH}$ ), short hinge, and part of the CH2 of the camel two-chain immunoglobulin IgG3,

- 5    - purifying the two shortest bands of 620 and 550 basepairs from agarose gel, for example by Gene Clean,
- recovering the amplified DNA fragments containing nucleotide sequences encoding the V<sub>HH</sub> fragments,
- digesting the amplified products with restriction enzymes having target
- 10    sites within the amplified fragments and/or in the nucleotide primers, for example with PstI and BstEII,
- recovering the digested amplified DNA fragments,
- ligating the amplified DNA fragments to a phasmid vector, for example in a pHEN4 vector, in conditions allowing the expression of the amplified
- 15    fragments when the obtained recombinant vector is used to transform a host cell,
- transforming a determined bacterial host cell for example an E. Coli cell with the obtained recombinant phasmid vector, and growing the cells on selective medium, to form a library,
- 20    - infecting the obtained library of recombinant host cells after culture in an appropriate selective medium, with bacteriophages, for instance M13K07 bacteriophages to obtain recombinant phagemid virions,
- harvesting the recombinant host cells, adsorbed with the bacteriophages,
- incubating the recombinant host cells in conditions allowing secretion of
- 25    recombinant phagemid virions particles containing the recombinant phasmid, for instance the pHEN4 phasmid packaged within the M13 virion.
- isolating and concentrating the recombinant phagemid virions,
- submitting the phagemid virions to several rounds of panning with the antigen of interest previously immobilized, in conditions allowing the
- 30    adsorption of the phagemid virions on the immobilized antigen,
- eluting the adsorbed phagemid virions, and growing them on appropriate cells,
- amplifying the phagemid virions by infecting the cells with helper bacteriophage,

- 5    - recovering the virions and testing them for their binding activity against  
the antigen of interest, for example by ELISA,  
- recovering the phagemid virions having the appropriate binding activity,  
- isolating the nucleotide sequence contained in the phasmid vector and  
capable of being expressed on the phagemid virions as a V<sub>HH</sub> aminoacid  
10    sequence having the appropriate binding activity.

28.    Nucleotide sequence coding for a variable fragment V<sub>HH</sub> of a heavy  
chain of an immunoglobulin devoid of light chain, directed against an  
epitope of the tetanus toxin of Clostridium tetani, characterized in that it  
codes for an amino acid sequence according to claim 14 or 15.

- 15    29.    Nucleotide sequence coding for a variable fragment V<sub>HH</sub> of a heavy  
chain of an immunoglobulin devoid of light chain, directed against an  
epitope of the tetanus toxin of Clostridium tetani, characterized in that is  
comprises one of the following nucleotide sequences:

20

25

30

5

 $\alpha$ TT1

```
      10      20      30      40      50      60
      |      |      |      |      |      |
GAGGTGCAGCTGCAGGCGTCTGGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC

      70      80      90     100     110     120
      |      |      |      |      |      |
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG

      130     140     150     160     170     180
      |      |      |      |      |      |
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAACTAT

      190     200     210     220     230     240
      |      |      |      |      |      |
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT

      250     260     270     280     290     300
      |      |      |      |      |      |
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT

      310     320     330     340     350
      |      |      |      |      |
AGTGCCCGGTTCGGCTATGTACTGTGCGGGCCAGGGGACCCAGGTCACCGTCTCCTCA
```

$\alpha$ TT2

10 20 30 40 50 60  
GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC

70 80 90 100 110 120  
TCTTGACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT

130 140 150 160 170 180  
CCAGGAAAGGAGCGCGAGGGGGTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT

190 200 210 220 230 240  
TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT

250 260 270 280 290 300  
CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC

310 320 330 340 350 360  
GGTTGGCGAGGGCGGCAGTGGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG

370 380  
ACTCAGGTCACCGTCTCCTCA

5 30. Nucleotide sequence starting with nucleotide 440 and ending between nucleotide 489 and nucleotide 495 on the nucleotide sequence of figure 15 or this nucleotide sequence in combination with a nucleotide sequence according to anyone of claims 27 or 28.

31. Nucleotide sequence starting with nucleotide 440 and ending  
10 between nucleotide 479 and nucleotide 486 on the nucleotide sequence of figure 15 or this nucleotide sequence in combination with a nucleotide sequence according to anyone of claims 27 or 28.

32. Expression product in a host cell of a nucleotide sequence according to anyone of claims 28 to 31.

15 33. Nucleotide sequence encoding the constructs of anyone of claims 20 to 23.

34. Process for the preparation of monovalent bispecific DNA constructs encoding variable fragments of a heavy chain of an immunoglobulins which comprises the following steps:

20 a) ligating a nucleotide sequence coding for a variable  $V_{HH}$  fragment having a determined antigen- or epitope- specificity to a linker nucleotide sequence to form a  $V_{HH}$ -linker fragment;

b) ligating the formed nucleotide sequence coding for the  $V_{HH}$ -linker fragment to a nucleotide sequence coding for another  $V_{HH}$  fragment  
25 having a different antigen- and/or epitope-specificity,

wherein the linker sequence contains the nucleotide sequence coding for part of a hinge domain wherein the codons responsible for the dimerisation of the  $V_{HH}$  fragments especially by formation of a disulfide bridge between the last cysteine residues within the hinge domain are  
30 deleted.

35. Process according to claim 34 comprising one or several additional step(s) of ligation

36. Process according to claim 34 or 35 wherein the sequence encoding part of the hinge domain comprises or corresponds to the nucleotide  
35 sequence starting with nucleotide 400 and ending with one of the

- 5 nucleotides between nucleotide 479 and 486 of the nucleotide sequence on the sequence of figure 15.



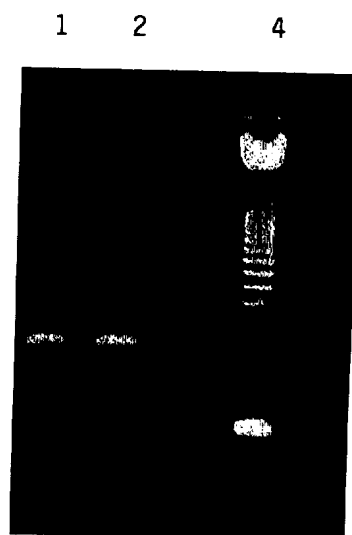


FIGURE 1

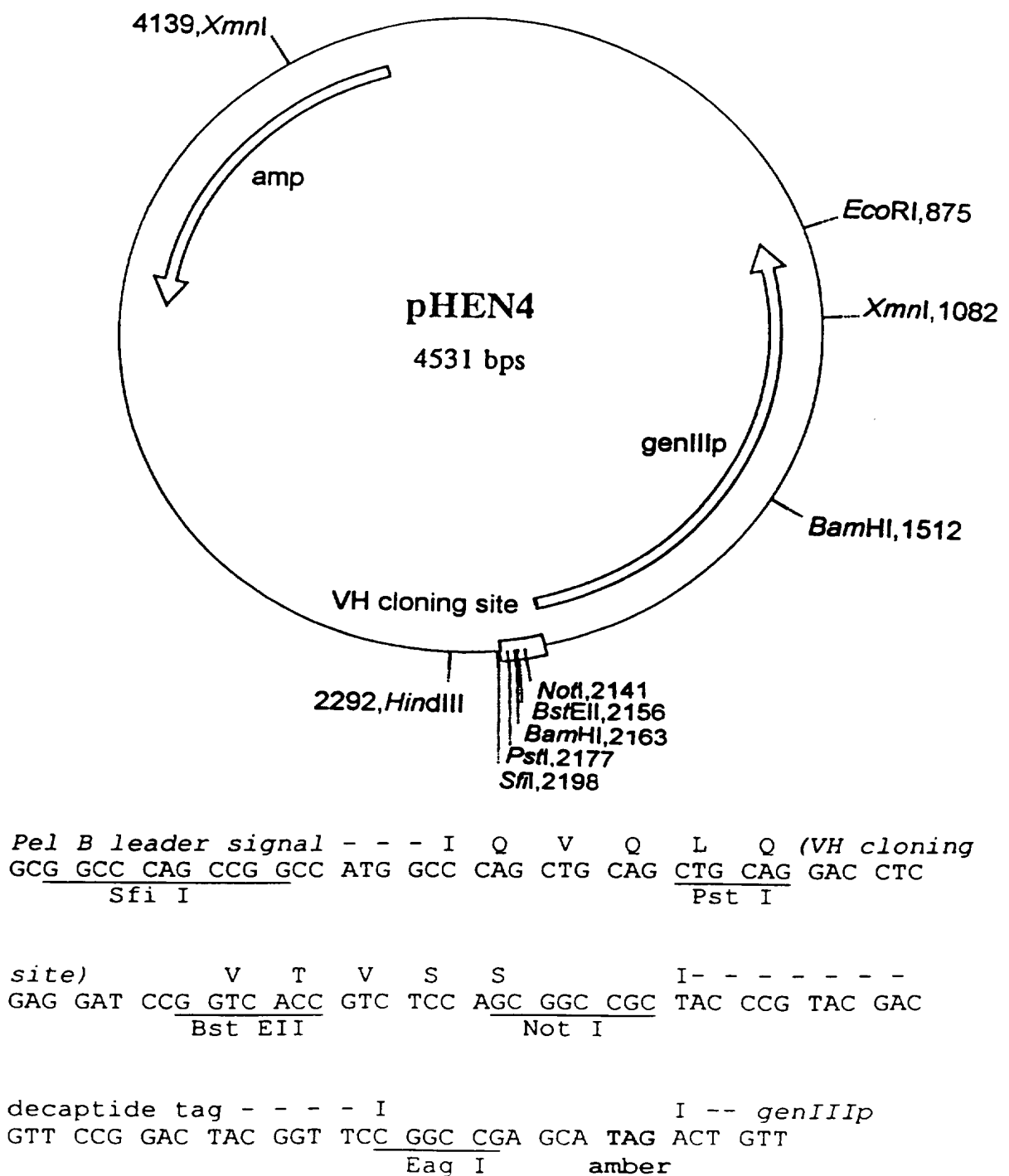


FIGURE 2

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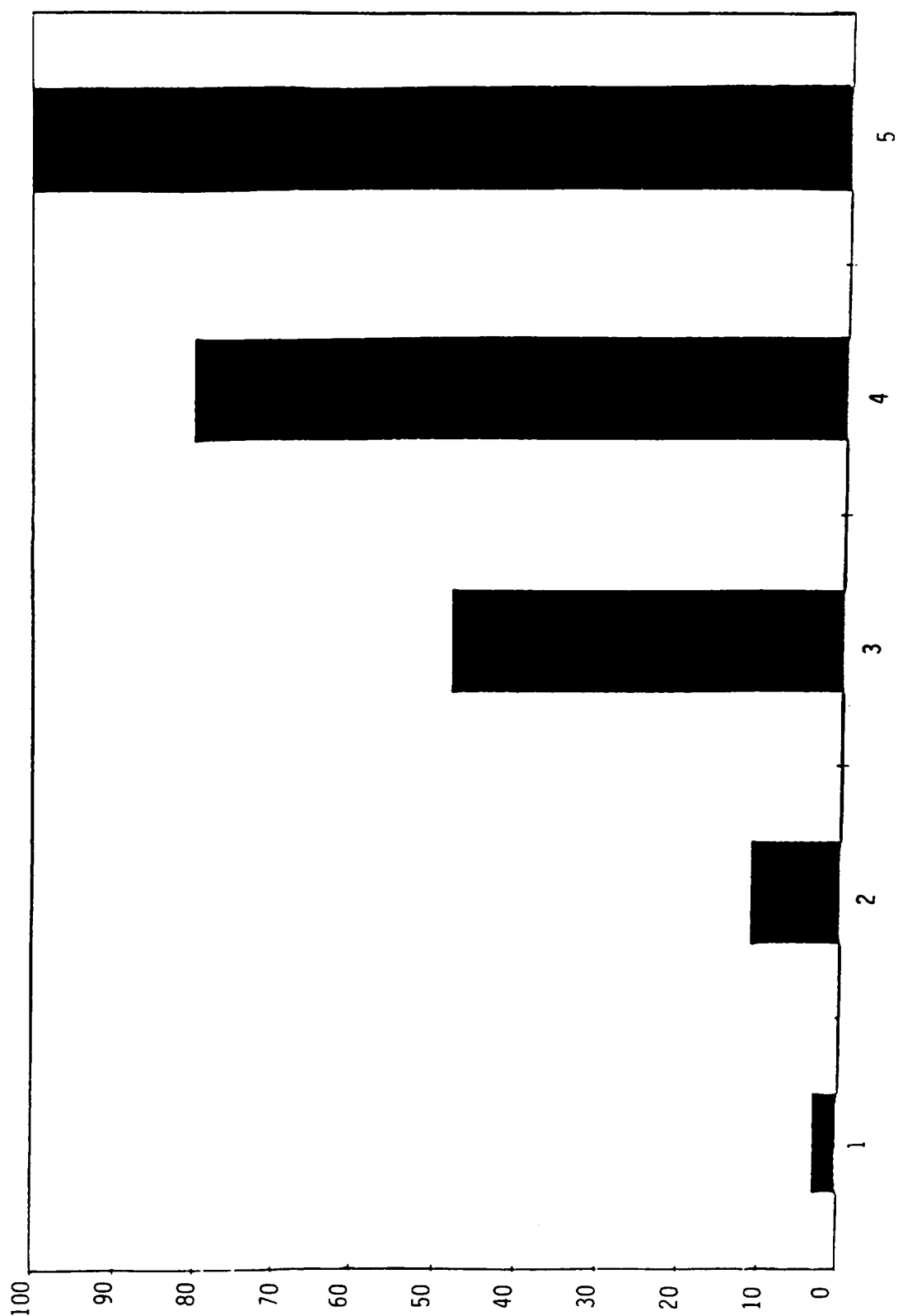


FIGURE 3

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pHEN4- $\alpha$  TT1 -----

10	20	30	40	50	60
GAGGTGCAGCTGCAGGCGTCTGGGGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGACTC					
GluValGlnLeuGlnAlaSerGlyGlyGly <u>Ser</u> ValGlnAlaGlyGlySerLeuArgLeu					
70	80	90	100	110	120
TCCTGTGCGGCCTCTGGGGGACAGACCTTCGATAGTTATGCCATGGCCTGGTTCCGCCAG					
SerCysAlaAlaSerGlyGlyGlnThrPhe <u>AspSerTyrAlaMETAla</u> Trp <u>Phe</u> ArgGln					
130	140	150	160	170	180
GCTCCAGGGAAGGAGTGCGAATTGGTCTCGAGTATTATTGGTGATGATAACAGAACTAT					
AlaProGlyLysGlu <u>Cys</u> GluLeuValSer <u>SerIleIleGlyAspAspAsnArgAsnTyr</u>					
190	200	210	220	230	240
GCCGACTCCGTGAAAGGCCGATTCACCATCTCCCGAGACAACGCCAAGAACACGGTATAT					
<u>AlaAspSerValLysGly</u> ArgPheThrIleSerArgAspAsnAlaLysAsnThrValTyr					
250	260	270	280	290	300
CTGCAAATGGACCGTCTGAATCCTGAGGACACGGCCGTGTATTACTGTGCGCAATTGGGT					
LeuGln <u>MET</u> AspArgLeuAsnProGluAspThrAlaValTyrTyrCysAlaGln <u>LeuGly</u>					
310	320	330	340	350	
AGTGCCCGGTGCGCTATGTAAGTGTGCGGGGCCAGGGGACCCAGGTCACCGTCTCCTCA					
<u>SerAlaArgSerAlaMET</u> TyrCysAlaGlyGlnGlyThrGlnValThrValSerSer					

FIGURE 4A

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pHEN4-  $\alpha$ TT2 -----

10 20 30 40 50 60  
GAGGTGCAGCTGCAGGCGTCTGGAGGAGGCTCGGTGCAGGCTGGAGGGTCTCTGAGGCTC  
GluValGlnLeuGlnAlaSerGlyGlyGlySerValGlnAlaGlyGlySerLeuArgLeu

70 80 90 100 110 120  
TCTTGTACAGCCGCTAATTACGCCTTTGATTCCAAGACCGTGGGCTGGTTCCGCCAGGTT  
SerCysThrAlaAlaAsnTyrAlaPheAspSerLysThrValGlyTrpPheArgGlnVal

130 140 150 160 170 180  
CCAGGAAAGGAGCGCGAGGGGGTTCGCGGGTATCAGTAGTGGTGGCAGTACCACAGCCTAT  
ProGlyLysGluArgGluGlyValAlaGlyIleSerSerGlyGlySerThrThrAlaTyr

190 200 210 220 230 240  
TCCGACTCCGTGAAGGGCCGATACACCGTCTCCCTTGAGAACGCCAAGAACACTGTGTAT  
SerAspSerValLysGlyArgTyrThrValSerLeuGluAsnAlaLysAsnThrValTyr

250 260 270 280 290 300  
CTACTGATAGACAACCTACAACCTGAAGACACTGCCATATACTACTGCGCAGGAGTGAGC  
LeuLeuIleAspAsnLeuGlnProGluAspThrAlaIleTyrTyrCysAlaGlyValSer

310 320 330 340 350 360  
GGTTGGCGAGGGCGGCAGTGCGCTGCTACTGGCAGAGACCTATCGGTTCTGGGGCCAGGGG  
GlyTrpArgGlyArgGlnTrpLeuLeuLeuAlaGluThrTyrArgPheTrpGlyGlnGly

370 380  
ACTCAGGTCACCGTCTCCTCA  
ThrGlnValThrValSerSer

FIGURE 4B

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1 2 3 4 5 6 7 8



FIGURE 5



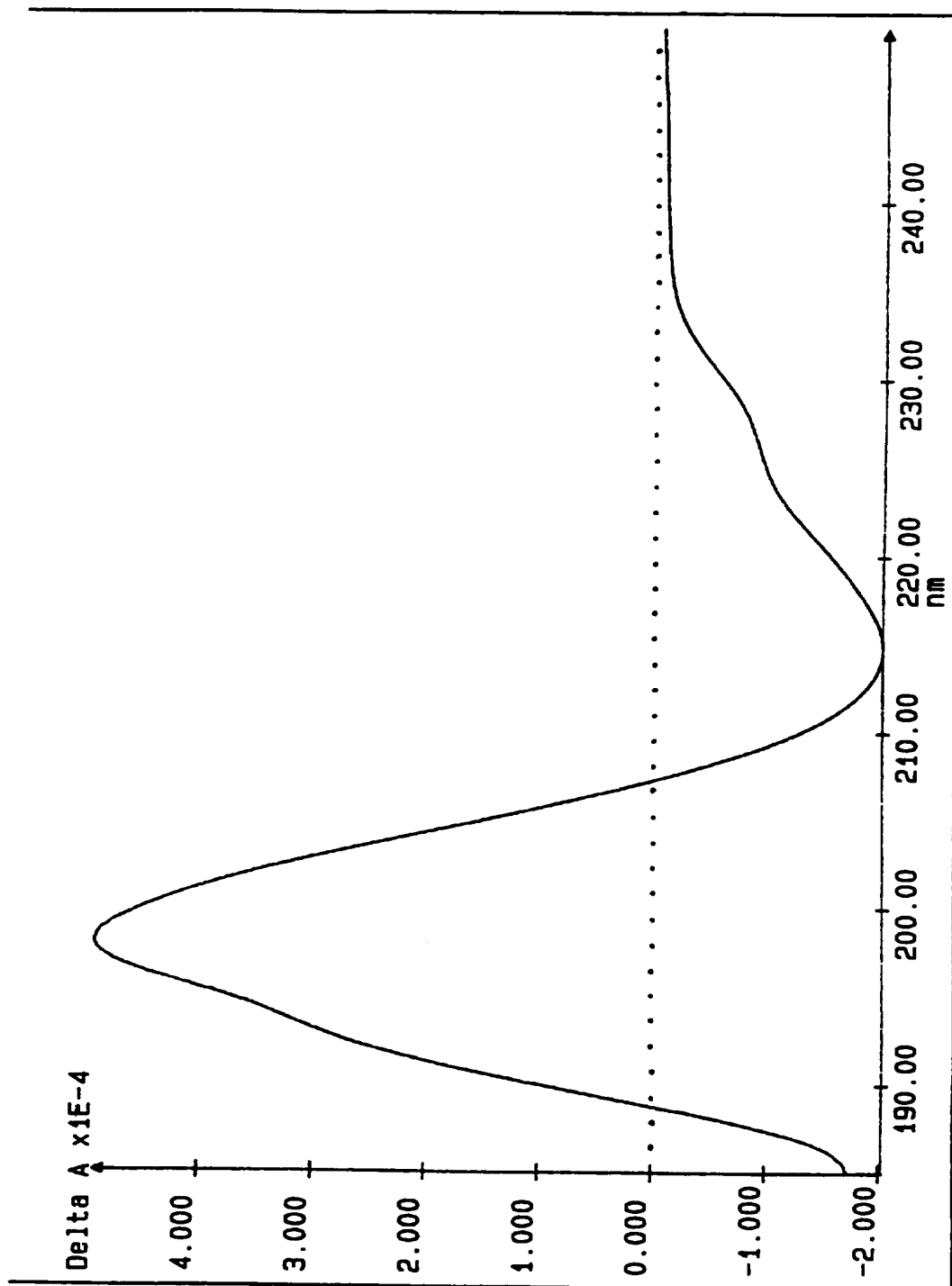


FIGURE 7



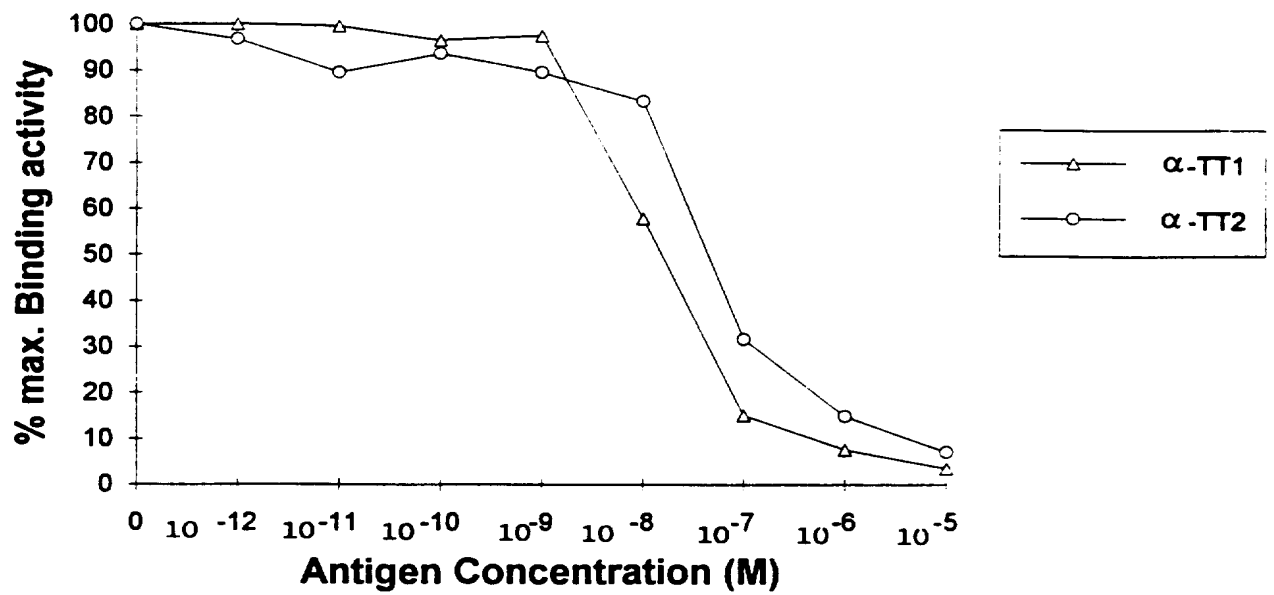
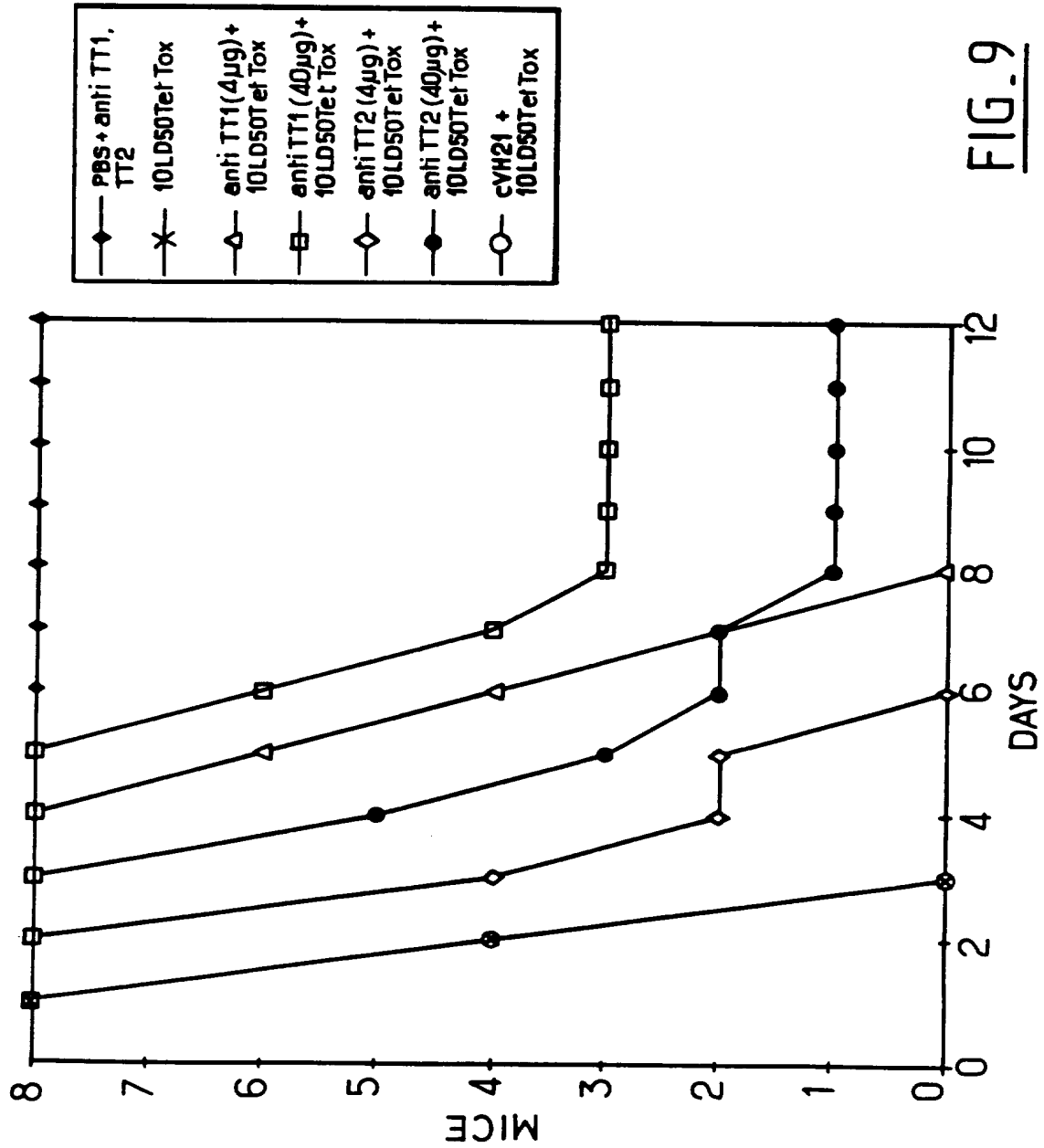
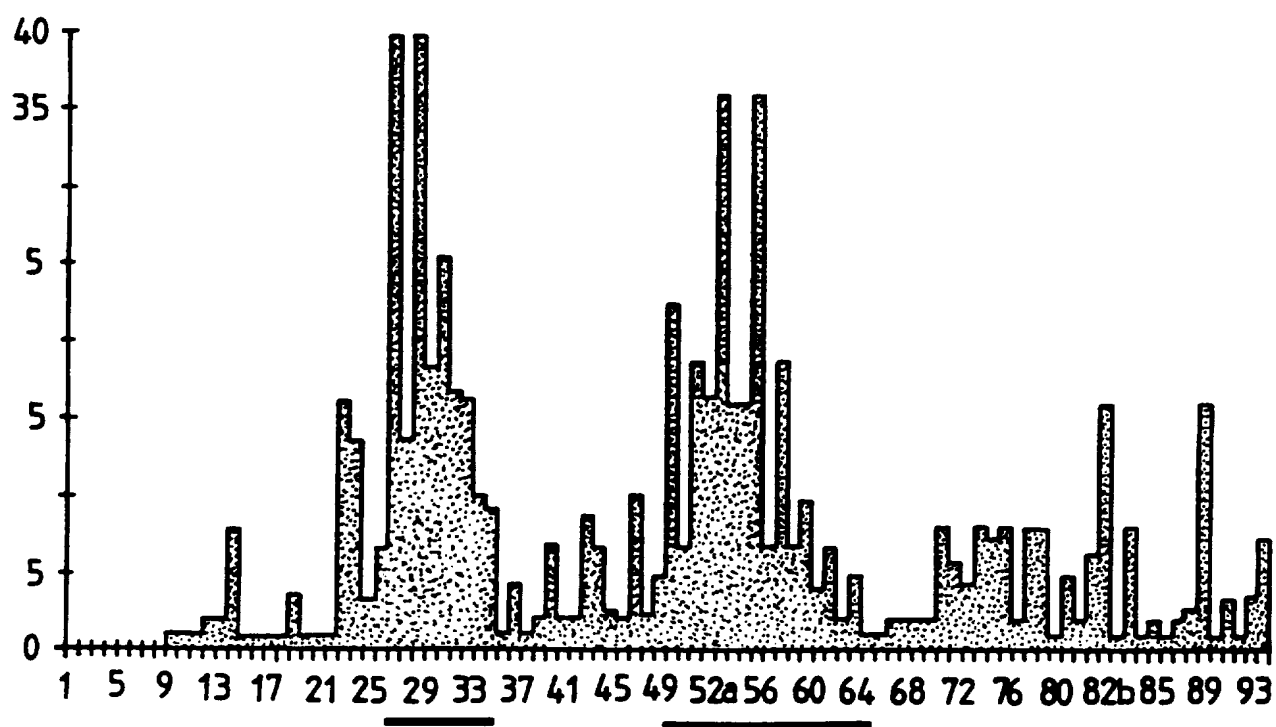


FIGURE 8

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FIG. 10

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GAG GTC CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA CAG 48  
 Glu Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gln  
 11

TCT CTG AGA CTC TCC TGT GCG ACC TCT GGA GCC ACC TCC AGT AGC AAC 96  
 Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Ala Thr Ser Ser Asn Ser

TGC ATG GGC TGG TTC CGC CAG GCT CCA GGG AAG GAG CGC GAG GGG GTC 144  
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val  
 37 44 45 47

CDR1

GCA GTT ATT GAT ACT GGT AGA GGG AAT ACA GCC TAT GCC GAC TCC GTG 192  
 Ala Val Ile Asp Thr Gly Arg Gly Asn Thr Ala Tyr Ala Asp Ser Val

CDR2

CAG GGC CGA TTG ACC ATC TCC TTA GAC AAC GCC AAG AAC ACG CTA TAT 240  
Gln Gly Arg Leu Thr Ile Ser Leu Asp Asn Ala Lys Asn Thr Leu Tyr

CTG CAA ATG AAC AGC CTG AAA CCT GAG GAC ACT GCC ATG TAC TAC TGT 288  
 Leu Gln MET Asn Ser Leu Lys Pro Glu Asp Thr Ala MET Tyr Tyr Cys

GCA GCA GAT ACA TCC ACT TGG TAT CGT GGT TAC TGC GGA ACA AAT CCA 336  
 Ala Ala Asp Thr Ser Thr Trp Tyr Arg Gly Tyr Cys Gly Thr Asn Pro

CDR3

AAT TAC TTT TCG TAC TGG GGC CAG GGG ACC CAG GTC ACC GTC TCC TCA 384  
Asn Tyr Phe Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr Val Ser Ser

FIGURE 11

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GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG 48  
 Asp Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala Gly Gly  
 11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC 96  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC 144  
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val  
 37 44 45 47

CDR1

GCA GCA ATT AAT ATG GGT GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG 192  
 Ala Ala Ile Asn MET Gly Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val

CDR2

AAG GGC CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT 240  
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT 288  
 Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys

GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT 336  
 Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly

CDR3

CTT TCC ACG GGA GGA TAT GGG TAT GAC TCC TGG GGC CAG GGG ACC CAG 384  
Leu Ser Thr Gly Gly Tyr Gly Tyr Asp Ser Trp Gly Gln Gly Thr Gln

GTC ACC GTC TCC TCA A 400  
 Val Thr Val Ser Ser

FIGURE 12

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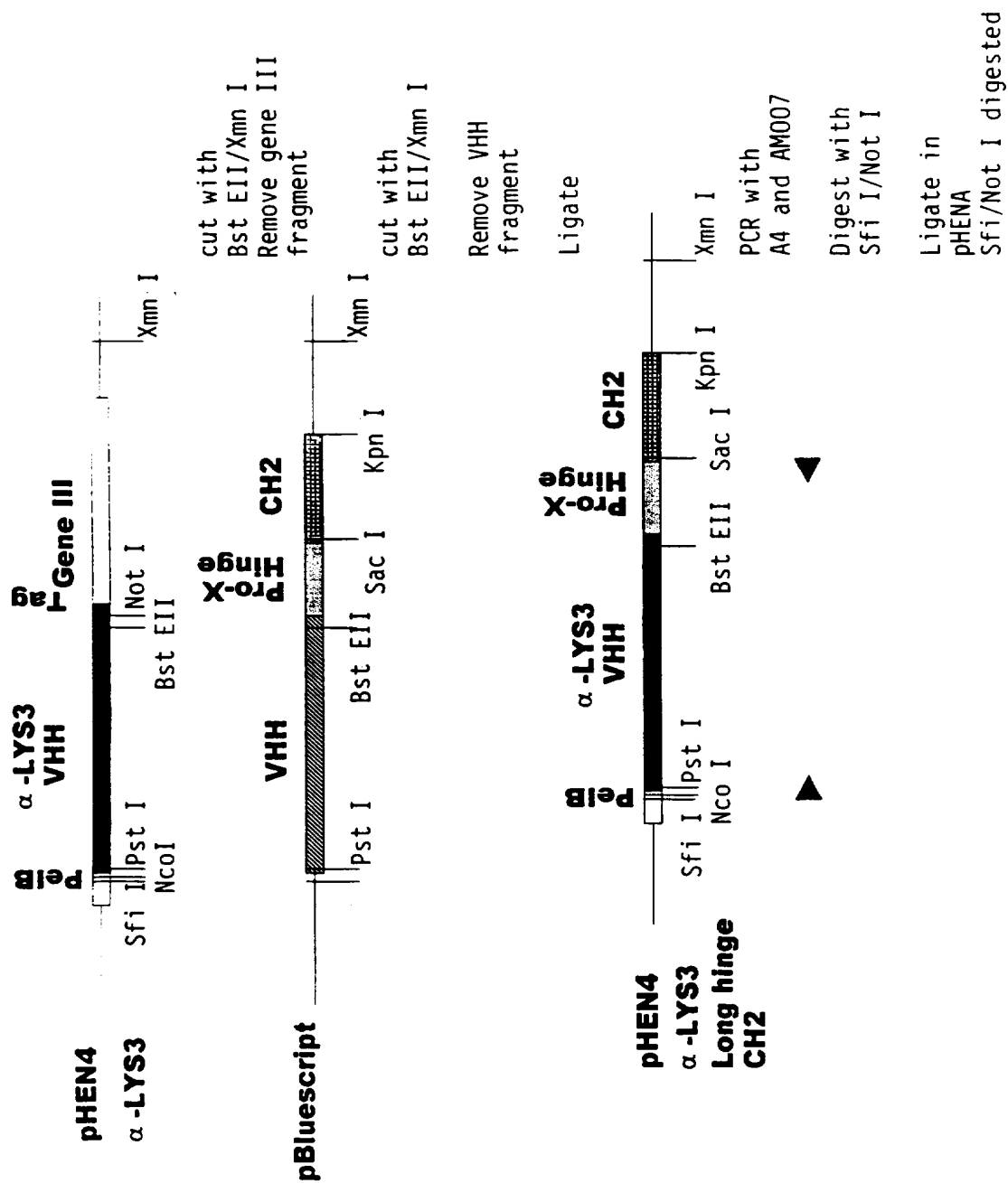


FIGURE 13A

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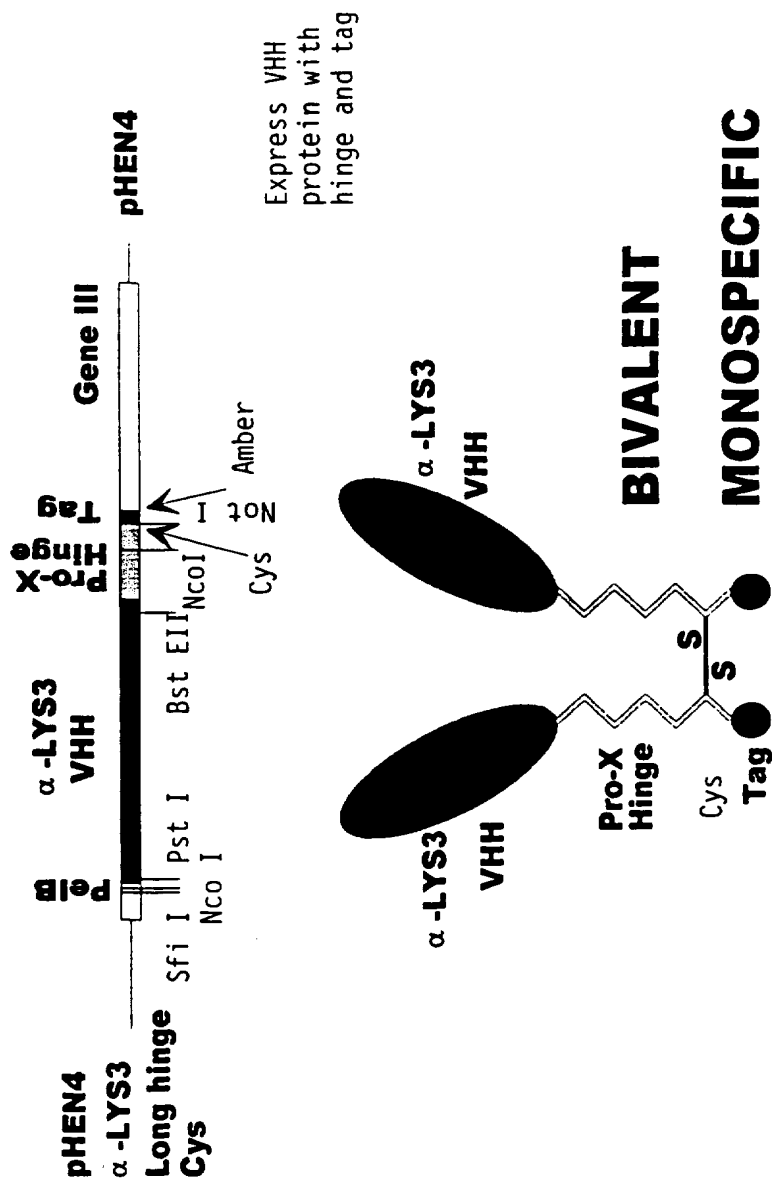


FIGURE 13B

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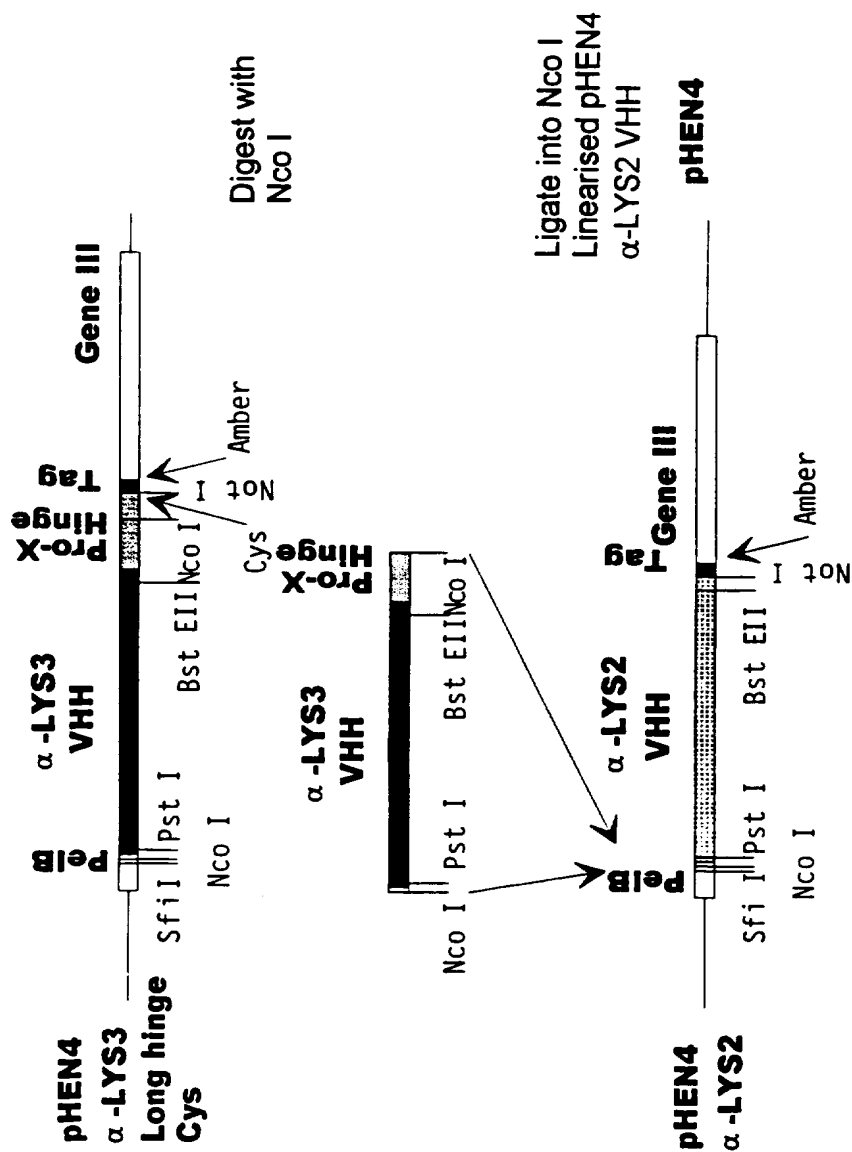


FIGURE 14A



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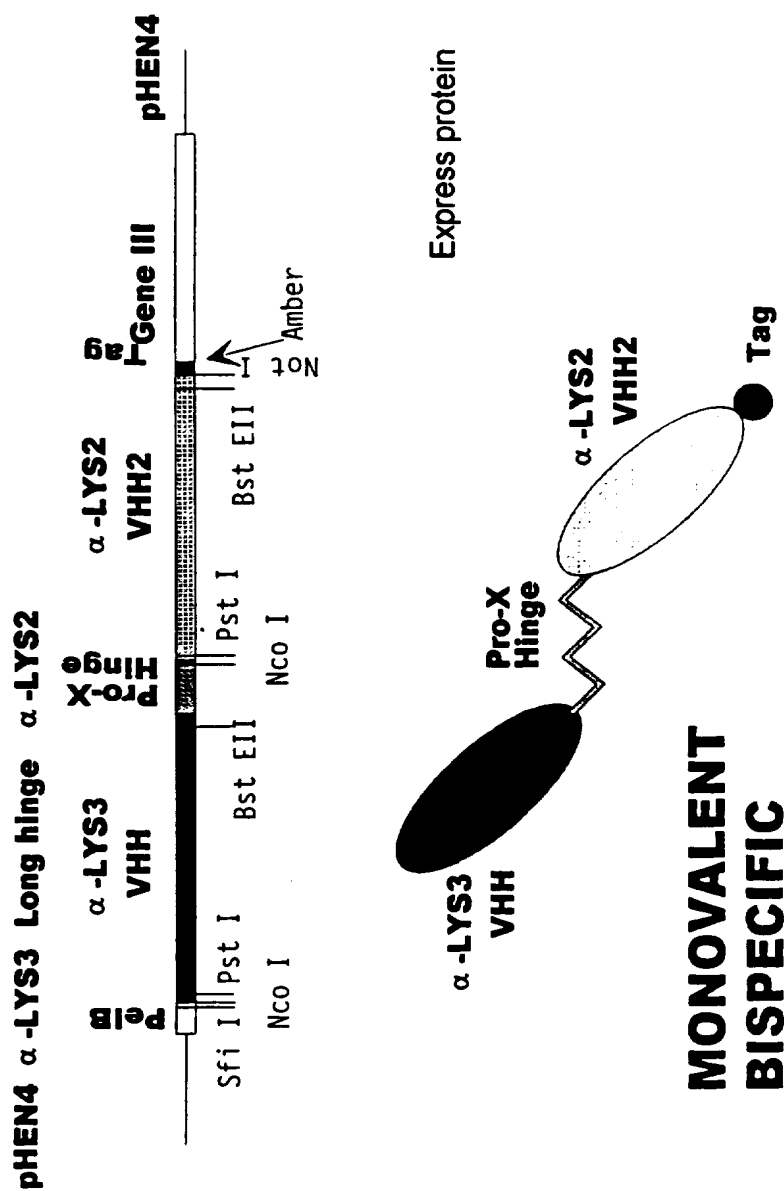


FIGURE 14B

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GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG 48  
 Asp Val Gln Leu Gln Ala Ser Gly Gly Ser Val Gln Ala Gly Gly  
 →α **LYS3** 11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC 96  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC 144  
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val  
 CDR1 37 44 45 47

GCA GCA ATT AAT ATG GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG 192  
 Ala Ala Ile Asn MET Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val  
 CDR2

AAG GGC CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT 240  
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT 288  
 Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys

FIGURE 15A

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336 GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT  
 Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly  
 CDR3

384 CTT TCC ACG GGA GGA TAT GGG TAT GAC TCC TGG GGC CAG GGG ACC CAG  
Leu Ser Thr Gly Gly Tyr Gly Tyr Asp Ser Trp Gly Gln Gly Thr Gln

432 GTC ACC GTC TCC TCA GAA CCC AAG ATA CCA CAA CCA CCA AAA CCA  
 Val Thr Val Ser Ser Glu Pro Lys Ile Pro Gln Pro Gln Pro Lys Pro  
 $\alpha$  LYS3  $\leftarrow$  HINGE  $\rightarrow$

480 CAA CCA CAA CCA CCA CAG CCA AAA CCA CCA AAA CCT GAA CCC  
 Gln Pro Gln Pro Gln Pro Lys Pro Gln Pro Lys Pro Glu Pro

528 ATG GAA TGC GGC CGC TAC CCG TAC GAC GTT CCG GAC TAC GGT TCC GGC  
 Nco I Not I  
 MET Glu Cys Gly Arg Tyr Pro Tyr Asp Val Pro Asp Tyr Gly Ser Gly  
 S TAG

537 CGA GCA TAG  
 Arg Ala ---

FIGURE 15B

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GAT GTG CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT GGA GGG 48  
 Asp Val Gln Leu Gln Ala Ser Gly Gly Ser Val Gln Ala Gly Gly  
 →αLYS3 11

TCT CTG AGA CTC TCC TGT GCA GCC TCT GGA TAC ACC ATC GGT CCC TAC 96  
 Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Ile Gly Pro Tyr

TGT ATG GGG TGG TTC CGC CAG GCC CCA GGG AAG GAG CGT GAG GGG GTC 144  
Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val  
 CDR1 37 44 45 47

GCA GCA ATT AAT ATG GGT GGT GGT ATC ACC TAC TAC GCC GAC TCC GTG 192  
 Ala Ala Ile Asn MET Gly Gly Gly Ile Thr Tyr Tyr Ala Asp Ser Val  
 CDR2

AAG GGC CGA TTC ACC ATC TCC CAA GAC AAC GCC AAG AAC ACG GTG TAT 240  
Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

CTG CTC ATG AAC AGC CTA GAA CCT GAG GAC ACG GCC ATC TAT TAC TGT 288  
 Leu Leu MET Asn Ser Leu Glu Pro Glu Asp Thr Ala Ile Tyr Tyr Cys

FIGURE 16A

336

GCG GCA GAT TCG ACC ATC TAC GCT AGT TAT TAT GAA TGT GGT CAC GGT  
Ala Ala Asp Ser Thr Ile Tyr Ala Ser Tyr Tyr Glu Cys Gly His Gly

CDR3

384

CTT TCC ACG GGA GAT GGG TAT GAC TCC TGG GGC CAG GGG ACC CAG  
Leu Ser Thr Gly Gly Tyr Glu Tyr Asp Ser Trp Gly Gln Gly Thr Gln

432

GTC ACC GTC TCC TCA GAA CCC AAG ATA CCA CCA CCA AAA CCA CCA  
Val Thr Val Ser Ser Glu Pro Lys Ile Pro Gln Pro Gln Pro Lys Pro  
 $\alpha$  LYS3  $\leftarrow$   $\rightarrow$  HINGE LINKER

480

CAA CCA CAA CCA CCA CAG CCA AAA CCA CCA AAA CCT GAA CCC  
Gln Pro Gln Pro Gln Pro Gln Pro Lys Pro Gln Pro Lys Pro Glu Pro  
LINKER HINGE  $\leftarrow$

528

ATG GCA GAG GTC CAG CTG CAG GCG TCT GGA GGA GGC TCG GTG CAG GCT  
Nco I  
MET Ala Glu Val Gln Leu Gln Ala Ser Gly Gly Gly Ser Val Gln Ala  
 $\rightarrow$   $\alpha$ LYS2 11

576

GGA CAG TCT CTG AGA CTC TCC TGT GCG ACC TCT GGA GCC ACC TCC AGT  
Gly Gln Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Ala Thr Ser Ser

FIGURE 15B

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624

AGC AAC TGC ATG GGC TGG TTC CGC CAG GCT CCA GGG AAG GAG CGC GAG  
Ser Asn Cys MET Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu

CDR1

37

44 45

672

GGG GTC GCA GTT ATT GAT ACT GGT AGA GGG AAT ACA GCC TAT GCC GAC  
Gly Val Ala Val Ile Asp Thr Gly Arg Gly Asn Thr Ala Tyr Ala Asp

47

CDR2'

720

TCC GTG CAG GGC CGA TTG ACC ATC TCC TTA GAC AAC GCC AAG AAC ACG  
Ser Val Gln Gly Arg Leu Thr Thr Ile Ser Leu Asp Asn Ala Lys Asn Thr

768

CTA TAT CTG CAA ATG AAC AGC CTG AAA CCT GAG GAC ACT GCC ATG TAC  
 Leu Tyr Leu Gln MET Asn Ser Leu Lys Pro Glu Asp Thr Ala MET Tyr

816

TAC TGT GCA GCA GAT ACA TCC ACT TGG TAT CGT GGT TAC TGC GGA ACA  
Tyr Cys Ala Ala Asp Thr Ser Thr Trp Tyr Arg Gly Tyr Cys Gly Thr

CDR3

864

AAT CCA AAT TAC TTT TCG TAC TGG GGC CAG GGG ACC CAG GTC ACC GTC  
Asn Pro Asn Tyr Phe Ser Tyr Trp Gly Gln Gly Thr Gln Val Thr Val

912

TCC AGC GGC CGC TAC GAC GTT CCG GAC TAC GGT TCC GGC CGA GCA TAG  
 Not I  
 Ser Ser Gly Arg Tyr Asp Val Pro Asp Tyr Gly Ser Gly Arg Ala ---  
αLYS2 TAG

FIGURE 16C

## INTERNATIONAL SEARCH REPORT

International Application No.

PC/EP 96/01725

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/13 C07K16/00 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 04678 (C. CASTERMAN ET AL.) 3 March 1994 cited in the application see claims	1-36
A	--- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 87, no. 20, WASHINGTON, DC, USA, pages 8095-8099, XP002011332 R. MULLINAX ET AL.: "Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library." cited in the application see the whole document --- -/-	1-6, 24-28

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search

21 August 1996

Date of mailing of the international search report

03.09.96

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## INTERNATIONAL SEARCH REPORT

International Application No.

PC/EP 96/01725

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROTEIN ENGINEERING, vol. 7, no. 9, OXFORD, GB, pages 1129-1135, XP002011333 S. MUYLDERMANS ET AL.: "Sequence and structure of Vh domain from naturally occurring camel heavy chain immunoglobulins lacking light chains." cited in the application see abstract see figures</p> <p>---</p>	11
A	<p>FEBS LETTERS, vol. 339, no. 3, 21 February 1994, AMSTERDAM, NL, pages 285-290, XP002011334 J. DAVIES ET AL.: "'Camelising' human antibody fragments: NMR studies on VH domains." see the whole document</p> <p>---</p>	11
A	<p>WO,A,93 01288 (DEUTSCHES KERNFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 21 January 1993 see the whole document</p> <p>---</p>	1,27
A	<p>WO,A,94 25591 (UNILEVER N.V.) 10 November 1994 see examples see claims</p> <p>---</p>	1-15, 24-32
A	<p>AMERICAN JOURNAL OF VETERINARY RESEARCH, vol. 50, no. 8, CHICAGO, IL, USA, pages 1279-1281, XP002011335 J. PAUL-MURPHY ET AL.: "Immune response of the llama (Lama glama) to tetanus toxoid vaccination." see abstract</p> <p>---</p>	4-6,28
P,A	<p>BIO/TECHNOLOGY, vol. 13, no. 5, USA, pages 475-479, XP002011336 J. DAVIES ET AL.: "Antibody VH domains as small recognition units." see the whole document</p> <p>-----</p>	1-15, 24-32



## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/EP 96/01725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9404678	03-03-94	EP-A- 0584421	02-03-94
		AU-B- 4949793	15-03-94
		CA-A- 2142331	03-03-94
		EP-A- 0656946	14-06-95
		FI-A- 950782	20-04-95
		JP-T- 8500487	23-01-96
		ZA-A- 9306086	22-07-94
-----			
WO-A-9301288	21-01-93	DE-A- 4122599	04-02-93
		EP-A- 0547201	23-06-93
		JP-T- 6500930	27-01-94
-----			
WO-A-9425591	10-11-94	AU-B- 6796094	21-11-94
		EP-A- 0698097	28-02-96
-----			